SYNTHETIC BIOLOGY

A Lab Manual

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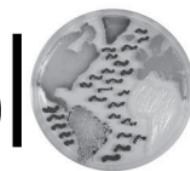
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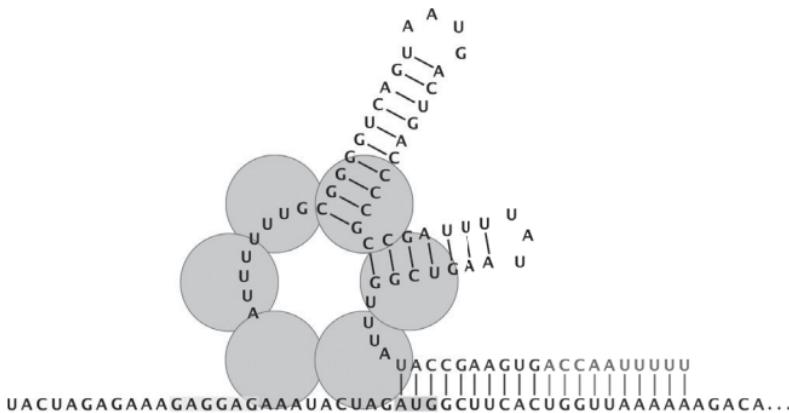
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World Scientific

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About the Authors

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Anthony C. Forster (M.D., Harvard; B.Sc.Hons., Ph.D. in Biochemistry, Adelaide) is a professor researching synthetic biology at the Department of Cell and Molecular Biology, Uppsala University, Sweden. He discovered the hammerhead catalytic RNA structure, authored patents that founded two biotech companies, edited synthetic biology volumes of *Methods* and *Biotechnology J.*, and created the synthetic biology lab course detailed in this manual.

Foreword

This foreword began when Tony Forster and I met a decade ago asking each other "What is Life?" (and "minimal life?") from the genetic, bioinformatics, and biochemical perspective. We soon began co-authoring a vision statement (eventually two articles) to accompany an experimental paper in the journal *Nature* which described the first assembly of genes (and a long operon) from oligonucleotides synthesized on and extracted from "glass chips" (the pattern and order of A, C, G, T photo-chemically programmed). More recently, I have participated (via Skype) in Tony's class on Synthetic Biology in Uppsala using the book *Regenesis* as a text (which I co-authored with Ed Regis). The question-and-answer sessions were very lively and thought-provoking, but there was no doubt that the field of Synthetic Biology needed at that time "A Lab Manual", such as the one that you now hold in your hands. The excitement of SynBio extends beyond even high school and college courses and competitions like iGEM … ramifying into the broad and unpredictable world of do-it-yourself biology. What garage holds the next Steve Jobs and Woz? What Lab Manual will be at their fingertips?

In addition to the definitions of synthetic biology in the Preface and Introduction, I'd add these: Synthetic biology is to early recombinant DNA as a genome is to a gene. The formation of SynBio as a discipline finally earns the label "genetic engineering." SynBio soars beyond making old biology "easier, " onward to also more accurate and effective goals. Biological engineering is poised to outdo all previous engineering fields because of three advantages: (1) SynBio has the ability to miniaturize in 3 and 4 dimensions via multiplexing and molecular "libraries", well exceeding the capabilities of 2-dimensional microfabrication of electrical engineers, or the multidimensional, yet coarse-grained, constructions of mechanical and civil engineers. (2) SynBio has inherited billions of years of evolutionary innovation and testing — an unparalleled list of parts, systems and applications. (3) SynBio can combine (1) and (2) to produce accelerated evolution, like the MAGE section of this book or "PACE" (phage-assisted continuous evolution). Clever selections and screens allow us to test not one prototype at a time, but billions. Moreover, we can use SynBio to evolve SynBio.

That auto-catalytic statement above brings us to the elephant (or mammoth) in the room — "Exponential technologies." The rate of change of biotechnologies (about 8-fold per year) has exceeded even the super rate of Moore's Law for electronics (about 1.5-fold per year). Keep in mind that these are not "laws" of physics, but trend lines and can change slope dramatically up or down. For example, DNA sequencing was going at about 1.5-fold per year from its start in 1968 until 2005, when "next-generation-sequencing" began.

Safety. Such exponential change, especially in the context of lowered costs and greater accessibility, raises

questions not just about conventional lab safety (as in Chapter 4 of this book), but potential for catastrophic scale bioerror or bioterror. As exhorted to the synthetic biologist Peter Parker by his uncle Ben (quoting Voltaire) "with great power comes great responsibility." Democratization of SynBio and open publishing of promethean protocols means that the vast majority of us who are virtuous and careful must be vigilant and creatively anticipate unintended consequences. It is not sufficient to say that we don't see any problem with our own work today.

The Future. This is the part of the foreword reaching forward. Forewarned is forearmed. This is intentionally very "meta", meaning self-referential. The book *Regenesis* was written into DNA and 70 billion copies made (easily). You may want to encode *Synthetic Biology: A Lab Manual* into DNA, or annotate your next genetic construct with your lab notes encoded into the genome. Synthetic biology is branching out into realms far beyond single microbial cells — to synthetic communities of such cells, synthetic ecosystems, synthetic multicellular systems, synthetic developmental biology, gene therapy, resurrecting extinct species, perhaps even changing our own species. Synthetic biology is not limited to cells or even to carbon-based chemistry (e.g. the intricate inorganic patterns of diatoms, bones, sea shells, magnetotactic particles in bacteria, silicon-based lenses and fibre optics in hexactinellid sponge). Biology inspires us to harness self-assembly, to turn industrial processes previously limited to enormous temperatures and toxic wastes to new "green chemistry" at 25 degrees Celsius. Protein/RNA-machines and DNA-origami represent, by far, the best nanotechnologies for rapidly designing and building atomically precise 3-dimensional objects, including mixtures of biopolymers and inorganic nanomaterials.

As electronics transitions from 2-dimensional to 3-dimensional and ventures down from "bulk" (15 nm) photolithography and dopants, attention is turning to molecular computing. Biomolecular systems are a million times denser and lower energy per digital operation. The central question is transitioning from "What can Synthetic Biology do?" to "Is there anything that Synthetic Biology will not impact?"

George Church
Harvard Medical School

Preface

Synthetic biology is an emerging multidisciplinary field with the potential to revolutionize our approach to global problems ranging from drug discovery to energy production. So what exactly is "SynBio," why is it so powerful, and why is it exciting students in ways never seen before? Perhaps the best answer is: synthetic biology is cutting-edge methods that make biology much easier to engineer. Yet there was no lab manual compiling these methods or detailing a synthetic biology lab course. Our goal was to fill this remarkable gap.

If you are one of the following: a teacher wanting to set up a student-driven synthetic biology lab course, a student taking such a lab course, a high school or tertiary institution student looking to participate in the international Genetically Engineered Machine (iGEM) competition, a new researcher in synthetic biology, or a non-specialist curious to know more about SynBio, iGEM and "BioBricks^{TM}," this book is for you. The protocols are straightforward enough to be used by any science students, be they biologists, chemists, engineers or even undifferentiated high school students.

Inspiration for writing this manual came from the grass roots level: students at Sweden's Uppsala University. They pressed the faculty to host five successive iGEM teams and to start a lab course, came up with the key project ideas, and thoroughly enjoyed themselves! The subject matter is also close to our hearts: ACF is a synthetic biology professor who created this lab course, JL is a Ph.D. student who set up and taught this lab, and EG is a Ph.D. student who tutored Uppsala's last three iGEM teams. So we finish with a warning before opening: SynBio is highly addictive!

Josefine Liljeruhm
Erik Gullberg
Anthony C. Forster

Acknowledgements

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Abbreviations and Acronyms

3A	3 antibiotic
°C	degrees Celsius
A	adenine
amp	ampicillin
ASD	anti-Shine Dalgarno sequence
ATP	adenosine triphosphate
b	base
BB	BioBrick™
bp	base pair
BSA	bovine serum albumin
BSL	biosafety level
C	cytosine
CDS	coding sequence
chloramp	chloramphenicol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddH$_2$O	doubly distilled water
dGTP	deoxyguanosine triphosphate
dH$_2$O	deionized water
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dTTP	deoxythymidine triphosphate
E. coli	*Escherichia coli*

EDTA	Ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
fMet	formylmethionine
FRT	flippase recognition target
FW	formula weight
G	guanine
GFP	green fluorescent protein
iGEM	international genetically engineered machine
kan	kanamycin
L, l	liter
LB	lysogeny broth
M	molar
m	milli (0.001x) or mass
MAGE	multiplexed automated genome engineering
mRNA	messenger RNA
MSDS	material safety data sheet
MW	molecular weight
N	nucleotide
n	nano (0.000000001x)
NAD	nicotinamide adenine dinucleotide
nt(s)	nucleotide(s)
OD	optical density
OE-PCR	overlap extension PCR
oligo(s)	oligodeoxyribonucleotide(s)
ORF	open reading frame
PACE	phage-assisted continuous evolution
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PNK	polynucleotide kinase
pol	polymerase
RBS	ribosome binding site
RFP	red fluorescent protein
RNA	ribonucleic acid

rRNA	ribosomal RNA
S	Svedberg unit
SD	Shine Dalgarno sequence
SDS	sodium dodecyl sulphate
SOB	super optimal broth
sRNA	small RNA
SynBio	synthetic biology
T	thymine
TATA box	core promoter sequence 5'-TATAAA
TBE	tris(hydroxymethyl) aminomethane borate ethylenediaminetetraacetate
T_m	melting temperature
Tris, Trizma^R	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
TSS	transcription start site
U	uracil or university
UV	ultraviolet
V, v	volume
x	times or multiplied by
μ	micro (0.000001x)

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1

Introduction

WHAT IS SYNTHETIC BIOLOGY, EXACTLY?

You may know it when you see it, but how would you
define it? Synthetic biology is undoubtedly a new, rap-
idly growing field that is captivating students and
researchers alike. Yet, like life itself, synthetic biology is
notoriously hard to define. Though the term "synthetic
biology" was coined a century ago, its use only came into
vogue one decade ago. This "renaissance" cannot be
attributed to any single breakthrough or publication, so
why did it occur? How does synthetic biology differ
from, for example, the older field of biotechnology that
encompasses DNA cloning, the polymerase chain reac-
tion (PCR), monoclonal antibodies and protein overex-
pression? Engineers may emphasize "**the development
of foundational technologies that make the design and**

construction of engineered biological systems easier"
(Endy, 2005), such as BioBricks™ (Knight, 2003).
Alternatively, biologists and chemists wishing to
encompass both *in vitro* and *in vivo* projects may
describe synthetic biology as "**the complex manipula-
tion of replicating systems**" (Forster and Church, 2007).
Still others define synthetic biology in terms of applica-
tions where, for example, **bioenergy, biomaterials and
biosensors** are synthetic biology while antibodies and
induced pluripotent stem cells are not. The use of engi-
neering principles for biological applications is not new,
as evidenced by the long-term success of biotechnology.
But what is definitely new versus classical recombinant
DNA and PCR is that synthetic biology is much easier
and more creative. The parts and techniques are more
standardized and cheaper, allowing faster, more modular
use with more predictable outcomes based upon more
precise measurements of activities. Computer-aided
design, analysis and modeling have further hastened
progress. These next-generational technologies, together
with tagged libraries and inexpensive, rapid, commercial
oligodeoxynucleotide/gene syntheses and sequencing,
have empowered biology and engineering students and
scientists like never before.

THE iGEM OUTBREAK

iGEM is an acronym for **international Genetically
Engineered Machine** and is a worldwide annual compe-
tition in synthetic biology for students from secondary
and tertiary institutions. The Massachusetts Institute of
Technology (MIT) organized the first competition in 2004
between teams from five universities in the U.S.A.
Projects are student-driven and lab work is mostly done

during the summer when students and labs are free from classes. iGEM has proven to be one of the most motivational educational methods ever devised, with the competition growing every year to now encompass 230 teams, including 30 teams in a high school division (Fig. 1).

Fig. 1 iGEM teams competing at MIT in 2006. Annual iGEM competitions have since expanded to encompass thousands of students worldwide. (Photograph by Randy Rettberg; taken from Wikipedia with permission.)

There is every reason to expect iGEM will continue expanding until it reaches most major educational institutions in the world. In parallel with this infectious, grassroots movement, career opportunities in synthetic biology in industry and academia are ballooning, mandating more defined education in synthetic biology than achievable just through iGEM. Thus, student enthusiasts are teaming up with university administrators to demand the creation of formal courses in synthetic biology, with our full lab course beginning in 2013 as a result. Formal courses complement and differ from iGEM by providing a more rounded education, requiring individual responsibility for knowledge and lab skills, and awarding creditation on an individual basis.

A SYNTHETIC BIOLOGY LAB MANUAL

Even the most highly motivated synthetic biology students in general, and iGEM students in particular, can be pretty raw in their knowledge and require considerable guidance with lab work. And teachers with no prior experience in synthetic biology can be pressed with the daunting task of setting up an entire lab course from scratch. At the time of writing, there are some synthetic biology protocols available online (*e.g.* see Lab section 4 of Chapter 5), but what is sorely lacking is a lab manual. Here we address this unmet need with three goals:

1. To provide **teachers and lab managers** with all the information they need to set up and run a synthetic biology lab course that spans from 2 to 5 weeks or more of full-time work;

2. To provide **high school students and tertiary institution students and researchers** with protocols for lab work; and

3. To provide **iGEM students** with practical information on setting up and running their own summer project in a host lab.

The underlying philosophy of our lab course design is similar to that of iGEM: to foster learning through student-driven, creative research with cutting-edge methods in small teams. Teams encourage learning through discussions and teamwork, not to mention being a practical way of economizing use of reagents and equipment. **Students will not only learn key synthetic biology technology such as BioBrick™ cloning, but also have the opportunity to create their own projects with varying difficulties.** Where the course philosophy differs from iGEM is in requiring each individual student to submit their own lab book for assessment and also to take a final exam. Without this individual responsibility, some team members will rely too heavily on other team members or become too specialized, thus failing to learn the important principles and failing to keep up with all aspects of the project. For example, an iGEM computational modeler may not understand operon function.

Just as iGEM teams struggle each year to pick a good project, so did we agonize in selecting a project for the lab course. Key considerations are outlined here as they may be helpful to future iGEM teams and course planners alike.

1. Expensive new equipment should not be required due to budget limitations.
2. *In vivo* replicating systems are generally easier to engineer in a novel way than *in vitro* ones. And most synthetic biology is *in vivo*.
3. *Escherichia coli* (*E. coli*) is the best model organism in biology. It is the best characterized, among the simplest and fastest growing, safe, and it is compatible

with by far the most BioBrick™ DNA parts (already numbering in the thousands).

4. The use of chromoproteins as easy readouts of gene expression is encouraged. Chemical substrates are not required, neither is the ultraviolet (UV) light that is needed for detecting the related fluorescent proteins such as green fluorescent protein (GFP). Furthermore, the colors can be changed readily by mutagenesis to become lighter, darker or even different colors. The advent of several chromoproteins that can be manipulated easily in *E. coli* using BioBricks™ is new, and we knew from our iGEM team that students really enjoyed making colored bacteria! For these reasons we selected a chromoprotein expression project for our lab course.

2

Genes, Chromoproteins and Antisense RNAs

Use of this manual requires a little basic knowledge in chemistry and biochemistry. Such principles are well covered by many textbooks (see References), including the only textbook on the principles of synthetic biology published at the time of writing (Freemont and Kitney, 2012). The reader is encouraged to consult these textbooks for the structures of biological macromolecules and their functions such as base pairing and catalysis. Nevertheless, it is difficult to find updated practical information in three rapidly evolving fields central to our experimental design: codon bias, chromoproteins and antisense RNAs in *E. coli*. Before covering these three topics below, some basic aspects of molecular biology directly relevant to synthetic biology and our lab are introduced.

E. coli DNA: CHROMOSOMES, PLASMIDS AND COPY NUMBER

E. coli is a bacterium growing in our colons and is the only "chassis" organism used in this manual.

Chassis

When the word "chassis" is used in synthetic biology, it simply refers to the organism that will be used to host the synthetic system. The most used and best characterized chassis of them all is *E. coli*. There are many different strains of *E. coli*, each having advantages and disadvantages. For cloning and assembly, cloning strains like DH5α are used because they give high transformation frequencies and good plasmid DNA preparations. However, for expressing synthetic devices, a healthier wild-type strain like MG1655 is more suitable than the rather weak and somewhat slower-growing cloning strains. While many synthetic biology standards and assembly methods are chassis independent, others are not (*e.g.* promoters and codon bias). It is very important to bear this in mind when moving a gene from one chassis into another.

E. coli contains a single, double-stranded, circular, chromosomal DNA molecule of ~4.6 million base pairs encoding ~4400 genes. Some *E. coli* cells also maintain much smaller, double-stranded, circular DNA molecules of a few thousand base pairs known as plasmid DNAs (Fig. 2).

Plasmid DNA is easier to engineer than chromosomal DNA in many ways. For example, while the copy number of chromosomal DNA per cell is one, the copy
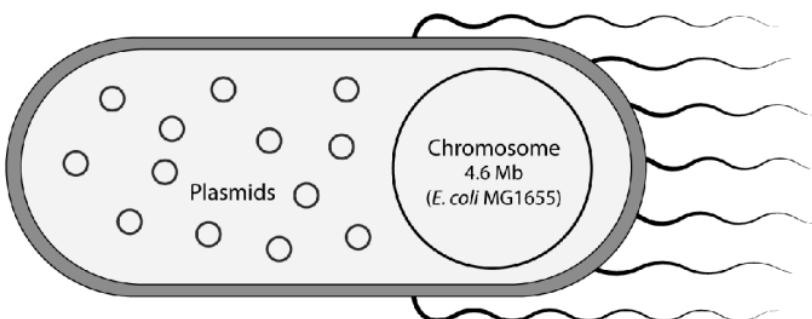
Fig. 2 *E. coli* cell containing chromosomal and plasmid DNAs.

number of a plasmid per cell may be anywhere from one to hundreds, depending on the particular origin of replication on the plasmid DNA. Thus one way to increase expression of a gene on a plasmid is to increase the copy number of the plasmid, thereby increasing the number of gene copies per cell.

Plasmid copy number and compatibility

A plasmid's host range, compatibility with other plasmids and copy number is determined by its origin of replication. Only one plasmid from each compatibility group can be stable in a strain at a time, so if you want to design a multi-plasmid system (*e.g.* for our antisense system), the plasmids must come from different compatibility groups. The difference between expressing a certain construct from the bacterial chromosome (~1 copy), from a medium-low copy plasmid (~15 copies) or from a high copy plasmid (500 copies) can be dramatic. To get high enough expression from a low or medium copy plasmid, a strong promoter may be required. However this promoter on a high copy plasmid could give such high expression that it would be toxic to the cell.

COUPLING OF TRANSCRIPTION AND TRANSLATION IN BACTERIA

The central dogma of molecular biology dictates that DNA encodes RNA which encodes protein (Fig. 3).

The particular type of RNA that encodes protein is called messenger RNA (mRNA). RNA synthesis from a DNA template is catalyzed by RNA polymerase and is termed transcription. Protein synthesis from an mRNA template is catalyzed by ribosomes and is called translation. In contrast to higher organisms where transcription is physically separated from translation, bacteria couple transcription and translation (Fig. 4).
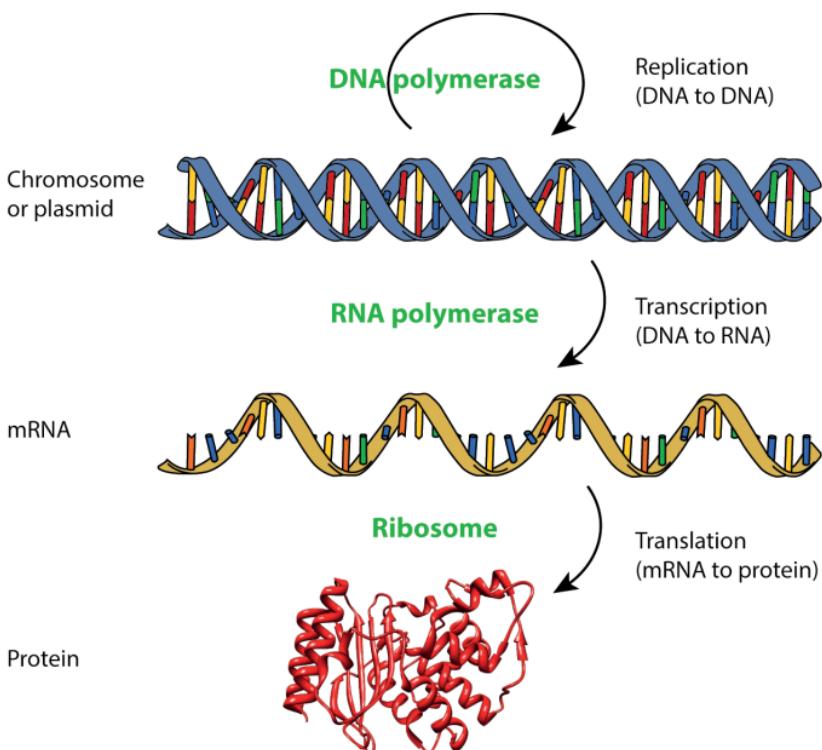
Fig. 3 The central dogma of molecular biology. The top and middle structures are taken from Wikipedia with permission.
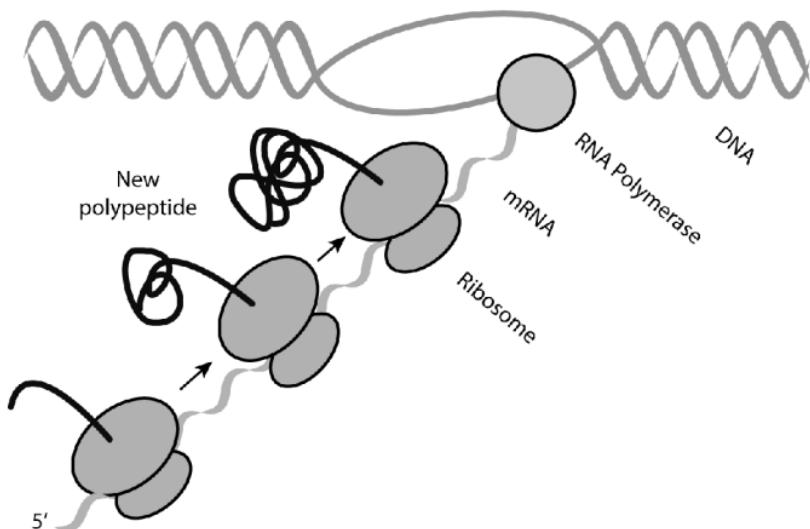
Fig. 4 Transcription and translation are coupled in bacteria.

A practical implication for synthetic biology of this coupling is that translation can feed back on transcription very rapidly. So rapidly, in fact, that even the initial speed of the translating ribosome can determine whether or not the mRNA it is translating is synthesized completely or terminated prematurely (termed attenuation).

Some RNAs are not translated; these are termed stable RNAs or non-coding RNAs. Examples include the ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) involved in translation, and the small regulatory RNAs engineered as part of the lab course (see below).

PROMOTER AND TERMINATOR FOR TRANSCRIPTION

Initiation of transcription is determined by protein-DNA interactions. The proteins are called RNA polymerase and transcription factors. The DNA portion is called the promoter and lies at the upstream (5′) end of the gene(s)

to be transcribed. In bacteria, one promoter may initiate transcription for one or several genes, an arrangement termed an operon (Fig. 5).

In experiments described in this manual, expression levels of RNAs and proteins are often changed by using different DNA promoter parts, leaving the polymerase and transcription factors unchanged. In *E. coli*, the primary (housekeeping) transcription factor is called sigma-70. The complex between sigma-70 and RNA polymerase recognizes promoter sequences with the bipartite consensus shown (Fig. 5). The TTGACA box lies at −35 nucleotides from the transcription start site (TSS) and the TATA (Pribnow) box at −10. The TATA sequence corresponds to one of the least stable double-stranded DNA secondary structures, thereby facilitating the opening
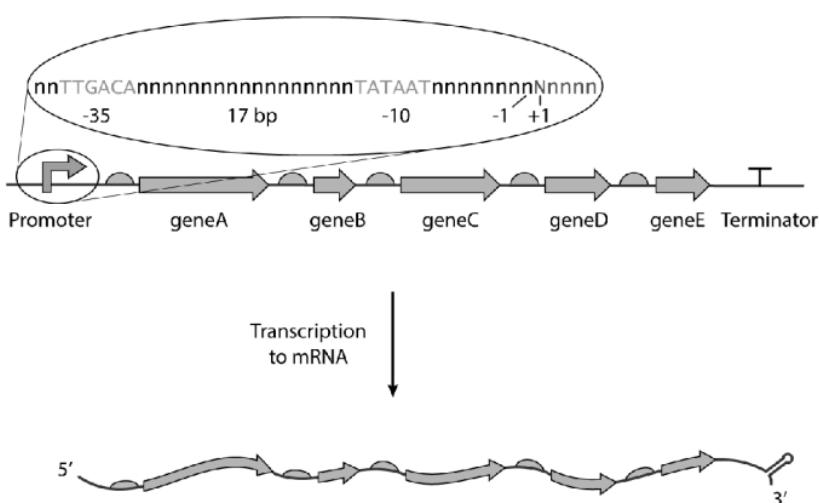
Fig. 5 Consensus *E. coli* promoter sequence upstream of an operon encoding five genes. N is any nucleotide, and there is no nucleotide numbered 0. Semi-circles represent ribosome binding sites (parts are represented according to the Synthetic Biology Open Language visual standard at http://www.sbolstandard.org/visual).

(melting) of the double helix necessary for transcription initiation.

Additional protein-binding sequences are often incorporated into the promoter region in synthetic biology to regulate transcription. Sequences called operators bind repressor proteins while enhancer sequences bind activator proteins. The activities of both types of proteins can be controlled with small molecules called inducers.

Termination of transcription occurs when the polymerase synthesizes an RNA sequence containing a stem-loop structure immediately followed by a run of U bases (Fig. 6).

Termination structures facilitate dissociation of the nascent RNA transcript from its DNA template because of low stability of the DNA-RNA hybrid. Terminators are placed between genes to insulate them from each other by stopping the RNA polymerase from continuing to transcribe from one gene into the next. However, some
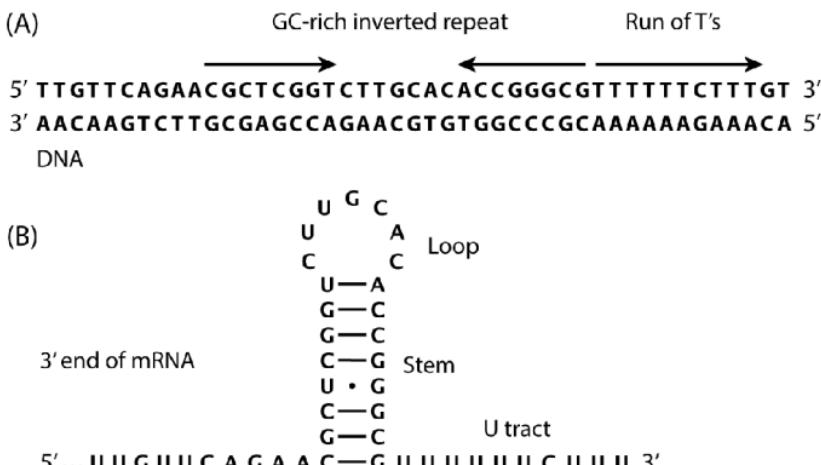
Fig. 6 (A) DNA sequence encoding a transcription termination signal. **(B)** Secondary structure of the terminator RNA sequence encoded in A. Additional AU and GU base pairs are possible.

leakiness into adjacent genes is to be expected because terminators are not 100% efficient.

The presence of a terminator stem-loop structure also increases the half-life of the mRNA, which is another potential control point for synthetic biology. But in practice, altering mRNA stability is difficult to do in a predictable, modular manner. Nevertheless, it is important to bear in mind that mRNAs are generally degraded by ribonucleases very rapidly in bacteria as this enables fine temporal control of gene expression by switching off transcription.

RIBOSOME BINDING SITE (RBS)

Initiation of translation in bacteria is primarily determined by base pairing. The first step is the binding of the small subunit of the ribosome to the RBS (also called the Shine–Dalgarno sequence), a sequence in the mRNA 5–8 bases upstream of the AUG start codon (Fig. 7A).

The RBS base pairs with the 3' end of the 16S rRNA in the ribosomal small subunit (anti-Shine–Dalgarno sequence, ASD), with the efficiency of base pairing determining the efficiency of initiation of translation. This efficiency is dictated not only by the strength of the base-pairing hybrid but also by the availability of the RBS for base pairing. Single-stranded RBSs bind to the ASD most efficiently, but usually the RBS is sequestered somewhat by weak or strong base pairing in *cis* with nearby sequences in the mRNA (Fig. 7B). Thus, while there are several BioBrick™ RBS parts available, the efficiency of translation initiation from each will vary depending on the context of where the RBS is inserted.
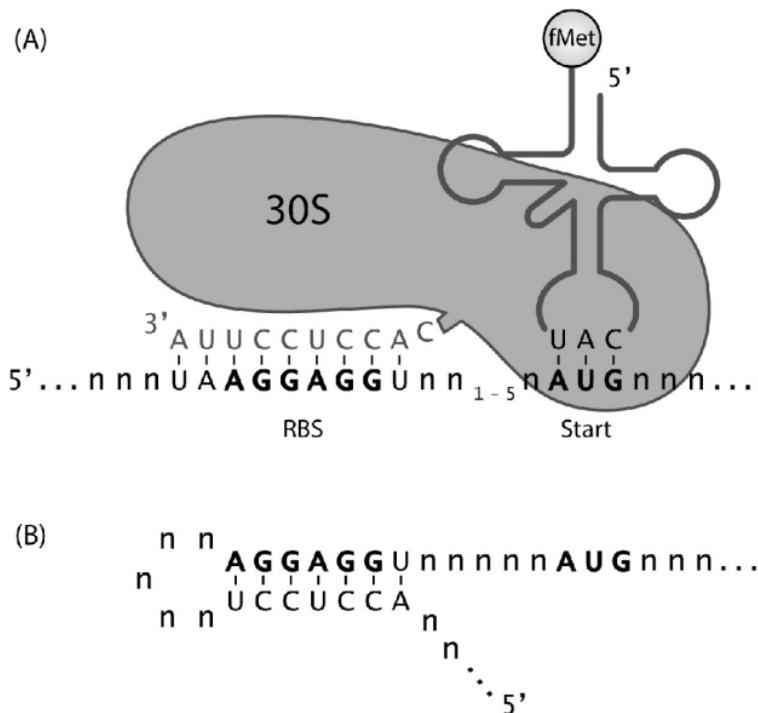
Fig. 7 Initiation of translation and inhibition of initiation. (**A**) For initiation of translation, first the RBS in the mRNA base pairs with the 30S subunit of the ribosome. Then a nearby downstream mRNA sequence termed the start codon base pairs with initiator tRNA charged with formylmethionine. For simplicity, initiation factors 1, 2 and 3 are not shown. (**B**) mRNA secondary structure involving the RBS inhibits binding of the 30S subunit and hence initiation.

The second step in translation initiation is base pairing between the start codon (almost always AUG) and the CAU anticodon of initiator fMet-tRNA$^{\text{fMet}}$ (Fig. 7A). The efficiency of this step is not affected by mRNA sequences directly adjacent to the start codon, but it is decreased substantially by using an alternative start codon, GUG, that pairs less stably with the initiator tRNA anticodon.

CODON BIAS

Once the fMet-tRNA^{fMet} is correctly positioned at the start codon of the mRNA by the ribosomal small subunit and accessory initiation factor proteins, the large (50S) ribosomal subunit binds to form the 70S ribosome. The ribosome then catalyzes translation of the next triplet codon into its cognate amino acid via base pairing with a cognate aminoacyl-tRNA. That amino acid is added onto fMet to form a nascent peptide, and this peptide is then extended one amino acid at a time until the ribosome reaches a stop codon and terminates translation. There are only 20 different, proteinogenic, elongator amino acids encoded by 61 different codons in the universal genetic code, so most of the 20 amino acids are encoded by more than one codon (termed synonymous codons; Fig. 8).

		2nd base				
		U	**C**	**A**	**G**	
5' base	**U**	Phe Phe Leu Leu	Ser Ser Ser Ser	Tyr Tyr Stop Stop	Cys Cys Stop Trp	U C A G
	C	Leu Leu Leu Leu	Pro Pro Pro Pro	His His Gln Gln	Arg Arg Arg Arg	U C A G
	A	Ile Ile Ile Met	Thr Thr Thr Thr	Asn Asn Lys Lys	Ser Ser Arg Arg	U C A G
	G	Val Val Val Val	Ala Ala Ala Ala	Asp Asp Glu Glu	Gly Gly Gly Gly	U C A G

Fig. 8 The universal genetic code.

Interestingly, different codons for the same amino acid are used non-randomly in genes: there is always codon usage bias within synonymous codons, termed codon bias.

Codon bias varies dramatically between different organisms and organelles and even varies at different segments within one gene. Explanations for codon bias are varied and still being revised (Forster, 2012; Goodman *et al.*, 2013). However, there is no doubt that codon bias was selected by multiple different evolutionary forces such as mutational bias, GC richness of DNA, RNA secondary structure and efficiency of translation. In fast-growing bacteria such as *E. coli*, translational speed is likely the main driver of codon bias. Genes from foreign organisms (*e.g.* chromoprotein genes) often express poorly in *E. coli* because some of the required cognate aminoacyl-tRNAs are relatively less abundant in *E. coli* than in the natural host. Indeed expression can often be improved by adding tRNA genes to *E. coli* or by changing the codon bias of the coding sequence to that of *E. coli*. For the latter engineering approach, a table of codon bias in the most highly expressed proteins (Fig. 9) is more useful than a table of the overall codon bias of the *E. coli* chromosome (not shown).

Gene synthesis companies and free websites offer the use of programs for codon optimization (as well as DNA and mRNA structure optimization), but the rules are still unclear. In theory, protein expression levels should only be affected by the rate of initiation, not the rate of elongation. So the only codons needing optimization should be the initial ones, as only they affect the rate of clearance of the ribosome from the initiation region of the mRNA. Yet codon optimization is usually performed across the entire gene. This latter approach is probably necessary

		2nd base					
		U	C	A	G		
5' base	U	7.92 **UUU** F phenylalanine 23.25 **UUC** F 2.73 **UUA** L leucine 4.27 **UUG** L	16.33 **UCU** S serine 11.68 **UCC** S 1.98 **UCA** S 2.51 **UCG** S	6.72 **UAU** Y tyrosine 16.52 **UAC** Y 4.18 **UAA** stop 0.00 **UAG** stop	2.76 **UGU** C cysteine 3.81 **UGC** C 0.19 **UGA** stop 7.03 **UGG** W tryptophan	U C A G	3' base
	C	3.86 **CUU** L leucine 4.09 **CUC** L 0.82 **CUA** L 60.75 **CUG** L	4.38 **CCU** P proline 1.09 **CCC** P 5.18 **CCA** P 28.82 **CCG** P	6.78 **CAU** H histidine 14.21 **CAC** H 7.01 **CAA** Q glutamine 27.28 **CAG** Q	43.82 **CGU** R arginine 20.59 **CGC** R 0.67 **CGA** R 0.62 **CGG** R	U C A G	
	A	15.79 **AUU** I isoleucine 43.86 **AUC** I 0.52 **AUA** I 21.67 **AUG** M methionine	20.64 **ACU** T threonine 26.70 **ACC** T 2.61 **ACA** T 4.17 **ACG** T	5.61 **AAU** N asparagine 29.21 **AAC** N 55.01 **AAA** K lysine 17.22 **AAG** K	2.19 **AGU** S serine 9.31 **AGC** S 0.63 **AGA** R arginine 0.03 **AGG** R	U C A G	
	G	43.18 **GUU** V valine 7.67 **GUC** V 22.31 **GUA** V 14.98 **GUG** V	39.49 **GCU** A alanine 11.81 **GCC** A 24.87 **GCA** A 24.11 **GCG** A	19.27 **GAU** D aspartic acid 33.74 **GAC** D 57.86 **GAA** E glutamic acid 16.97 **GAG** E	45.55 **GGU** G glycine 34.17 **GGC** G 1.26 **GGA** G 2.36 **GGG** G	U C A G	

Fig. 9 Codon usage frequencies (1×10^{-3}) in abundant proteins in *E. coli*. The standard genetic code format is used and initiator codons are omitted. Adapted from Forster (2012).

for massive overexpression to prevent ribosome "traffic jams" throughout the mRNA, but is it important for most synthetic biology applications which do not involve massive overexpression?

CHROMOPROTEINS

In nature, many organisms have bright colors caused by a wide spectrum of colorful pigments. While many of these pigments are small molecules produced by metabolic pathways, some of these colors come from pigmented proteins, chromoproteins. Most of these proteins get their color via a pigmented prosthetic group, *e.g.* heme bound in cytochromes or hemoglobin. Here, we focus on a different type of proteins, the family of fluorescent proteins and chromoproteins that are common in corals, sea anemones and jellyfish (Dedecker *et al.*, 2013). The most famous protein from this class is **green fluorescent protein (GFP)** from the jellyfish *Aequorea victoria*. These proteins do not require any prosthetic groups, but instead the chromophores are formed through reactions between side chains of the protein itself (S65, Y66 and G67 in the case of GFP; single-letter abbreviations of the amino acids are given in Fig. 9) in a form of post-translational modification called maturation. As GFP-type proteins can fold and mature properly in almost any organism without exogenous cofactors and they are encoded by single, small genes (~700 bp), they have become very useful as reporters in molecular and synthetic biology.

GFP-type proteins all share a similar beta-barrel structure, with the covalently bound chromophore protected inside the barrel (Fig. 10).
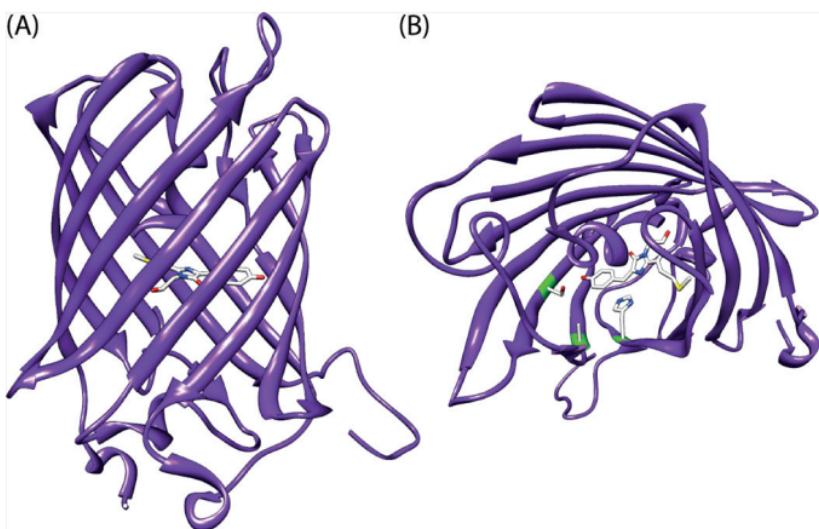
Fig. 10 Three-dimensional structure of chromoprotein asFP595 (also called asCP, KFP or asPink). (**A**) View illustrating beta-barrel structure with the covalently bound chromophore protected in the center. (**B**) View from a different angle that better shows the chromophore. Amino acid residues that affect the color of the chromophore are marked with green (A148, S165 and H203; numbering based on GFP). Rendering based on PDB 1XMZ.

The color of the protein is dependent on which amino acid residues form the chromophore as well as the interactions between the chromophore and the neighboring amino acid side chains inside the barrel. Many natural GFP-type fluorescent proteins have been engineered through both directed and random mutagenesis to generate a large number of novel proteins with new spectral properties, improved fluorescence, higher photostability, lower toxicity, increased pH tolerance and improved folding and faster maturation (Alieva *et al.*, 2008). The fluorescent proteins can have a wide range of colors: cyan, blue, green, yellow, orange and red. Most chromoproteins are blue, purple, pink or red, mature slowly and

are yet to be engineered. There are several useful fluorescent proteins and chromoproteins available in BioBrick™ format from the Registry of Standard Biological Parts (Table 1 in Appendices).

SMALL REGULATORY RNAs (sRNAs)

Regulation of gene expression in bacteria is often done post-transcriptionally via sRNAs, antisense RNA molecules that either increase or decrease mRNA translation. A typical sRNA is 50–400 bases long, and one sRNA can regulate several different mRNA targets. Many sRNAs are dependent on an RNA binding protein called Hfq that can both protect the sRNA from degradation and facilitate the interaction between the sRNA and its mRNA target (Sharma *et al.*, 2011).

Repression of gene expression via sRNAs usually involves binding to the RBS or the start of the coding region on the mRNA, hindering the ribosome from binding and thereby blocking initiation of translation (Fig. 11A).

There are also cases where the sRNA binds elsewhere on the target mRNA, and instead promotes degradation by recruiting ribonucleases (Fig. 11B). In cases where an sRNA activates translation of a gene, the sRNA often binds to the 5′ untranslated region of the mRNA to open up a structure that hid the RBS from the ribosome (Fig. 11C).

In synthetic biology, sRNAs show great potential for rapid fine tuning of gene expression (Na *et al.*, 2013) compared with standard approaches. For example, if there is only a single gene copy, a genetic knockout might be lethal and would not allow partial inhibition of the gene. And compared to transcriptional regulation via repressors, translational regulation via sRNAs has much
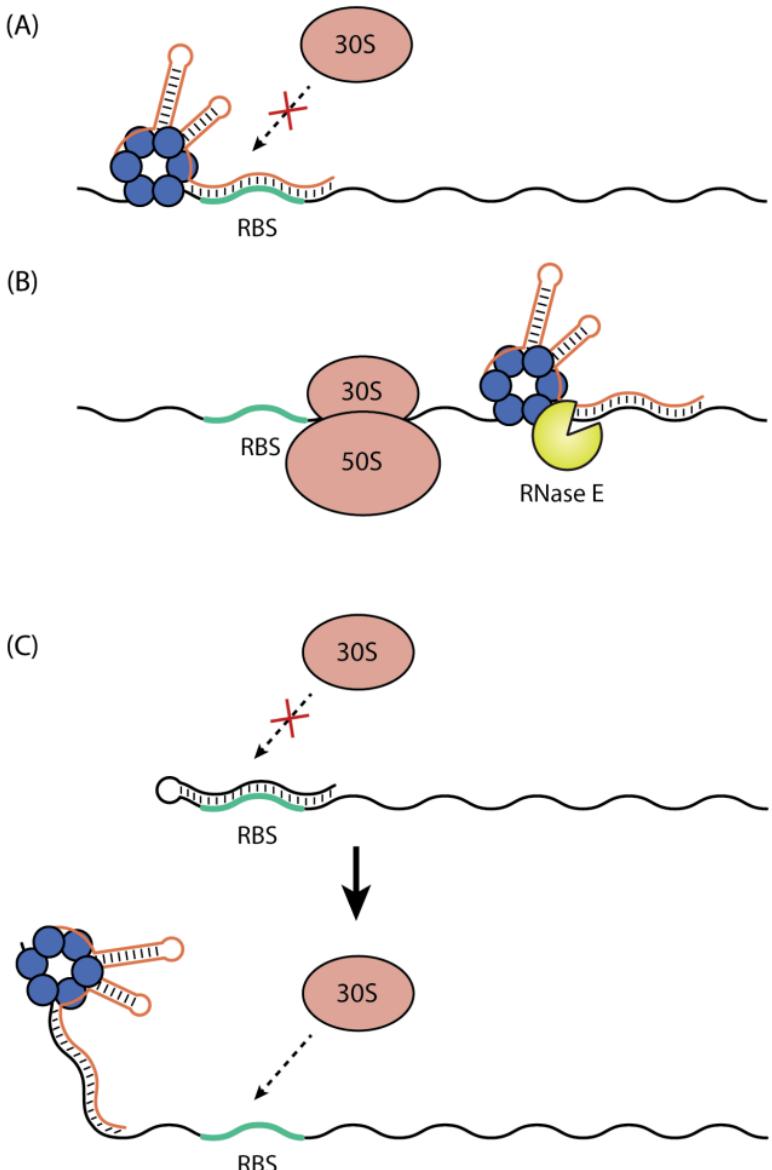
Fig. 11 Mechanisms of action of sRNAs. (**A**) An sRNA blocking initiation of translation by base pairing with the RBS on the mRNA. The blue circles represent Hfq protein. (**B**) An sRNA binding downstream on the mRNA, promoting degradation of the mRNA by recruiting ribonuclease. (**C**) Top, the 5′ untranslated region of the mRNA can contain structures that sequester the RBS from the ribosome. Bottom, binding of an sRNA to the 5′ untranslated region of the mRNA opens up the RBS so that the ribosome can bind and initiate translation.

shorter response times. A small RNA can start repressing its target immediately after transcription, while a repressor gene needs to be translated to a protein after transcription. Even then, the expression of the gene will continue as long as there is still mRNA remaining in the cell. sRNAs are also interesting tools for gene regulation since they are generally smaller and easier to construct than protein repressors. A common approach when designing synthetic sRNAs is to use the Hfq-binding portion of natural sRNAs, and then engineer the 5' antisense portion to bind the target mRNA (see Fig. 36 and top left of Fig. 50). This can be done either through rational design and secondary structure modeling or by random mutagenesis and screening. When designing synthetic sRNAs, it is important to keep their target specificity in mind. For example, RBSs tend to be quite similar to each other, so a synthetic sRNA complementary to an RBS would bind non-specifically to many different mRNAs, likely being toxic to the cells.

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3

Lab Rooms and Equipment

THE PHYSICAL LAB SPACES

Our theory and lab course in synthetic biology in 2013 catered for 27 undergraduate students at Uppsala University. Lab work was mainly conducted in a single room measuring 8 x 13 meters, containing 40 meters long of discontinuous bench space, easy-to-clean surfaces and chairs, and standard safety features (Fig. 12). Identical rooms were used by our iGEM teams of up to 26 students per summer.

Adjacent to the main lab room was a room containing spectrophotometers (Figs. 13 and 14) and large centrifuges, a room for storage and weighing of chemicals, and an autoclave machine room.

Fig. 12 Main lab room for 27 students.

Fig. 13 UV-visible spectrophotometer containing 1 mL sample and reference cuvettes.

Fig. 14 Nanodrop™ UV-visible spectrophotometer for µL samples. Compared with spectrophotometers for 1 ml cuvettes, Nanodrop™ is not more sensitive, but it does have the advantage of not requiring dilution of each sample before measurement.

Because of safety regulations forbidding eating and drinking in the labs, we had adjacent rooms for coffee/ tea, lunch and group study.

Though we had two teaching assistants and two main lab rooms assigned for the class, we found it more practical to have all 27 students filling one lab room to capacity instead of spreading between two rooms. This halved the number of introductions required, ensured that all students received the same information simultaneously and facilitated maintaining at least one teaching assistant in the lab room as a safety measure. The class was divided into eight groups of three or four students and the students and equipment were spaced to

minimize congestion. Thus, PCR machines (Fig. 15), gel equipment (Figs. 16 and 17), *etc.* were each spread into multiple stations.

EQUIPMENT

This lab course was designed to take advantage of standard teaching lab equipment to keep the budget low. **All required major and minor items are listed in Appendices** and special safety considerations are given in Chapter 4. Some instructions for students are given here:

Fig. 15 PCR thermocycler.

Fig. 16 Agarose gel electrophoresis.
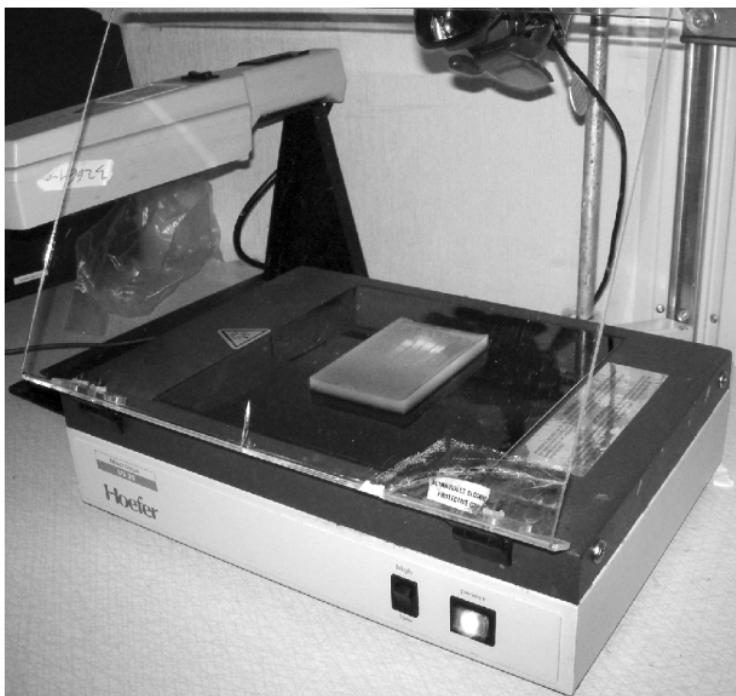
Fig. 17 UV light box imaging DNA bands stained with Sybr®Safe in an agarose gel.

Autoclave Machine

Because some bacteria survive boiling, sterilization of solutions requires heating aqueous solutions for 20 min at temperatures well above 100°C (typically 121°C). In order to prevent the aqueous solutions evaporating at such high temperatures, a steam atmosphere at high pressure is required, so water is initially added to the autoclave chamber (Fig. 18).

• Do not handle the autoclave machine without being instructed to do so.
• Only use autoclavable plastic, as other plastic can melt.
• Use distilled or double-distilled water to avoid lime deposits.
• Fill up only to 80% of the volume of a bottle, except for agar media for plates which are filled up to 60% of the bottle.
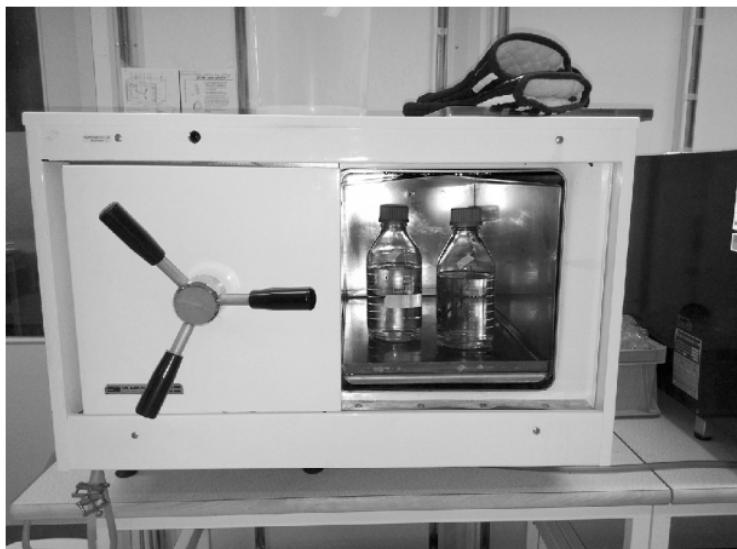
Fig. 18 Autoclave containing bottles with loosened autoclavable plastic caps.

- Loosen the cap of the bottle.
- Liquids are autoclaved for 20 min.
- Use insulated gloves when unloading.

Burners

These are essential for microbiology, both for sterilization and for preventing bacterial contamination. Burners cause upward air motion, thereby decreasing contamination of media. Great caution is required because the flames can be almost invisible and because of the danger of burns and fires.

- Use the correct gas container for the burner.
- Do not use plastic gloves while working near a flame.
- Be careful with glass spreaders and wire inoculation loops as both are fragile.
- See Chapter 4 (Fires section and Fig. 20 in Chapter 4) for additional safety advice on burners.

Gel Equipment

- Use gloves at all times because the equipment has been in contact with ethidium bromide and/or Sybr®Safe dyes.
- Be cautious when handling melted agarose — it is very hot.
- Add the comb straight after pouring the melted agarose into the tray.
- Make sure that the gel solidifies on a level surface.
- To prevent electric shock, do not apply voltage until the lid is properly placed upon the buffer chambers (Fig. 16).

Centrifuges

- If the centrifuge has a cooling system, the lid must not be left open or water will condense inside: treat it like your refrigerator!
- Use adaptors or a special rotor if centrifuging tubes for smaller volumes than 1.5 mL.
- Always balance the centrifuge rotor!
- Never leave the centrifuge until it has reached maximum speed — it should be shut down immediately if there is a loud noise!

Micropipettes

The micropipettes (or pipettemen) are key tools and it is important to keep them calibrated and use them properly.

- Never turn the knob past the printed volume range for that pipette. Consult the manufacturer's instructions for the exact range of your pipette.
- The largest error rate lies at the bottom of a pipette's range.
- Use the correct pipette tips for the volume being pipetted.
- Hold the pipette vertically while pipetting to ensure that roughly the right volume is aspirated (you will learn what looks right from experience).
- Aspirate the liquid carefully for accuracy and to avoid contamination of the pipette. This especially applies to cultures and non-viscous solvents like ethanol.
- Make sure that all of the volume leaves the tip.
- If a micropipette becomes contaminated or its knob is turned too far, give it to the lab assistant for cleaning or calibration.

Laboratory Bench

- Ensure that the table does not get too overcrowded with papers.
- Do not have any paper on the shelf over the lab bench as it is a potential fire hazard when using the burner. Never use paper bench coat when using a burner!
- Wash the bench with 70% ethanol prior to, and after, any work with cells and media.
- Clean the bench at the start and end of the day to avoid accumulation of paper and equipment that could be a source of bacterial contamination.

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4

Safety is Priority #1

The top priority in any lab must be safety. The risks are not insignificant as most seasoned scientists have either directly witnessed lab accidents or heard of their occurrence in their own institution. Risks can be minimized by understanding hazards and safety procedures, so safety training is mandatory before commencing lab work. Know where safety equipment and first aid kits are located. If an accident occurs, notify the lab assistant immediately.

FIRES

Everyone should become familiar with institutional fire regulations.

Responding to fires

The usual order of action is:

1. Save lives.
2. Call the fire brigade.
3. Alert people in the area.
4. Extinguish the fire if possible.
5. Close doors to the area.
6. Evacuate.
7. Reassemble outside the building at the designated meeting point.

Know the locations of the fire-fighting equipment, fire alarms and evacuation routes closest to the lab. A small fire can be extinguished quickly by smothering it in a fire blanket or by spraying it with a fire extinguisher. There is usually a choice of two kinds of fire extinguishers and the simplest choice is the one containing carbon dioxide, *i.e.* the one with a large nozzle (left of Fig. 19).

The carbon dioxide extinguisher is recommended for all types of fires, whereas the alternative liquid/foam fire extinguisher should not be used on electrical fires because the liquid stream can conduct electricity back to the firefighter!

Lab fires in biological labs are caused most commonly by the plating of cell cultures (Fig. 20).

Typically a metal or glass spreader is dipped into a glass container of ethanol, the ethanol on the spreader is then ignited by passage through a burner flame, and burning drops of ethanol inadvertently fall from the spreader either (i) onto paper bench coat covering the bench or (ii) into the glass container of ethanol with

Fig. 19 Fire extinguishers containing carbon dioxide (left) and liquid/foam (right).
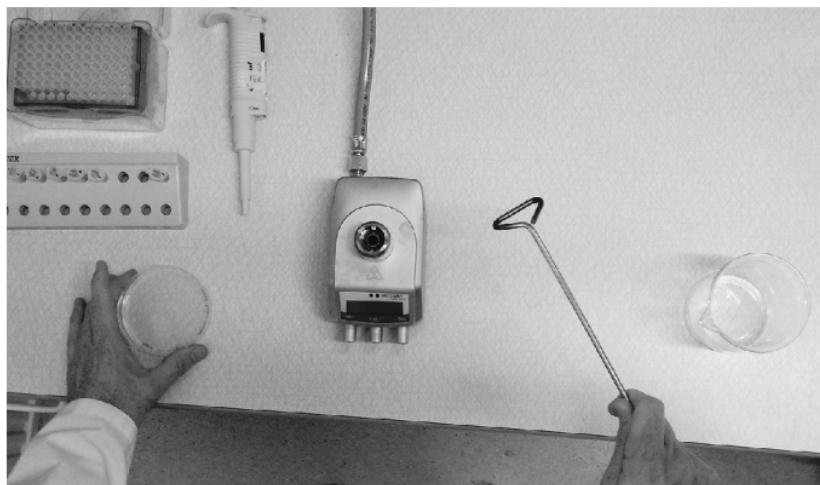
Fig. 20 Safe set-up for plating cell cultures.

the container possibly cracking due to the heat of the fire. To prevent this, one should:

1. Never use paper bench coat near burners.
2. Use a low reservoir volume of ethanol.

3. Place the ethanol reservoir at least a foot from the burner and on the opposite side of the burner from the plates (Fig. 20).

Other burner safety tips are:

4. Never wear plastic gloves when working with a flame.
5. Carry the gas container for the burner carefully with a firm grip. If it is dropped, make sure that there is no leakage by smelling for gas.
6. Notify the instructor of any gas leakage and do not light burners when there is a smell of gas.
7. Always light the match before opening the gas valve.
8. Avoid placing burners too close to overhanging shelves.
9. Never leave the table while a burner is on.

CHEMICALS

On each chemical container, there is a label that specifies the potential danger of the substance for humans and/or the environment. Chemicals should be handled cautiously with gloves, both for your safety and for decreasing the contamination risk. Always wear a lab coat and shoes as additional protection. Handling of liquid nitrogen requires a face mask and cryogenic gloves in a well-ventilated area, not a closed small room (Fig. 21).

For handling eye hazards such as hydrochloric acid, hydroxide solutions and organic solvents, safety glasses are required, and, depending on the quantities, also a fume hood (Fig. 21). This lab course is designed to minimize the use of such hazards and fume hoods (*e.g.* phenol is not required). Radioisotopes are also not used.

Upon chemical spillage, check your clothes and lab coat. Read the signs on the chemical container and the

Fig. 21 Safe pouring of liquid nitrogen. A fume hood for acids and solvents is in the background.

Material Safety Data Sheet (MSDS; available online) for further direction. Wipe off and wash the skin and notify the lab assistant. Know where the emergency eye wash and shower stations are and flush eyes immediately after contamination. In laboratories, it is not permitted to
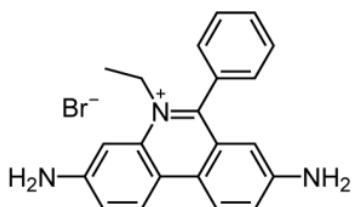
Fig. 22 Chemical structure of ethidium bromide dye. Taken from Wikipedia with permission**.**

mouth pipette, drink, eat or even chew gum. Storage and disposal of food and drink is also forbidden. Wash your hands when leaving the lab to prevent contamination of your face and food.

Ethidium Bromide

This dye is a potent mutagen that is widely used as a sensitive DNA stain in agarose gel electrophoresis. Its properties derive from its planar structure (Fig. 22) having a propensity to intercalate into double-stranded nucleic acids.

Because it is probably the most toxic chemical used in this manual and it requires special disposal procedures at most institutions, we strongly recommend the safer substitute, Sybr®Safe, for all labs (Fig. 17). The relative sensitivities of these two DNA stains are very similar. Always wear nitrile gloves when handling solutions of these stains as vinyl gloves are more permeable.

BIOLOGICAL SAFETY AND DISPOSAL

Whatever you wish to call the generation of new organisms by molecular methods, *e.g.* synthetic biology, recombinant DNA, molecular cloning or genetic engineering,

the field is regulated in most countries according to international biosafety guidelines:

1. CDC, Atlanta. Biosafety in Microbiological and Biomedical Laboratories.
   http://www.cdc.gov/biosafety/publications/bmbl5/bmbl5_sect_iv.pdf
2. WHO, Geneva. Laboratory Biosafety Manual, 2004.
   http://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf
3. ECDC, Directive 2000/54/ec of the European parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work (seventh individual directive within the meaning of Article 16(1) of Directive 89/391/EEC.
   http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2000:262:0021:0045:EN:PDF

   When working with microorganisms such as bacteria and viruses, there are four BioSafety Levels (BSL) numbered BSL1–4. This lab course is designed to be at BSL1, the lowest risk level. (The next lowest level, BSL2, involves pathogens that can, with low probability, cause moderate harm to people working with them, thus requiring special training.)

   The lab course includes expression of chromoproteins and antibiotic resistance proteins from plasmids transformed into well-categorized cloning strains of *E. coli*, *e.g.* DH5α. These cloning strains have low fitness outside the laboratory and are non-pathogenic.

   BSL1 requires decontamination of disposables, such as used tips and tubes, through steam autoclaving before disposal. Contaminated LB or SOB media must not be poured directly into the sink as they are environmental

hazards. After cell growth, cultures and culture waste products are sterilized with 0.01% iodine or dilute bleach for a couple of hours before sending the flasks for cleaning. Glass pipettes are sterilized by submersion in Virkon disinfectant solution for a couple of hours before rinsing and washing. Disposables such as tubes, pipette tips and gloves are sterilized by autoclaving before disposal. Sharp objects, like needles and broken glass, are handled carefully and disposed of in a puncture-resistant plastic container. There are strict rules to always wear laboratory coats when located inside the lab area. Protective eyewear is used when necessary, *e.g.* when there is risk for splashes or upon exposure to artificial UV radiation. Protective gloves must be changed upon contamination or if otherwise necessary. Before leaving the lab area, the workbench should be sterilized and hands washed.

DANGEROUS EQUIPMENT

Electric shocks can occur from mishandling equipment for agarose or polyacrylamide gel electrophoresis due to the high voltage differential between two exposed buffer reservoirs. Most modern commercial gel equipment has a built-in safety feature whereby attachment of the electrodes requires concomitant covering of the buffer reservoirs (Fig. 16). However older or "home-made" equipment may require labeling as hazardous. The simultaneous touching of both buffer reservoirs with different hands during electrophoresis must be avoided!

Centrifugation creates enormous forces on rotors, especially at the highest speeds and when rotors are imbalanced. Rotors also eventually develop metal fatigue and can crack. For these reasons, never leave a centrifuge until it has reached maximum speed so that you can turn

it off as soon as it begins to make a strange noise. This is important not only for safety, but also for preserving the equipment. As a general rule, dangerous and/or expensive equipment such as centrifuges, electrophoresis power packs, autoclaves and spectrophotometers should not be used without proper instruction.

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5

Lab Course Projects

TIME AND RESOURCES

As outlined in the introductory chapter, the goal of the lab course is to teach key synthetic biology technology and also to give students the opportunity to create their own projects with varying difficulties. Our course in spring 2013, detailed here in this manual, was five full-time weeks of laboratory work (Table 7 in the Appendices). Each day began with a one-hour lecture or tutorial on synthetic biology theory followed by the rest of the day in the lab.

While our schedule worked well according to the anonymous student evaluations, there is considerable flexibility in the protocols that allows adaptation to alternative scheduling requirements. Synthetic biology foundational technology has simplified molecular biology

so famously that novices, even high school students and do-it-yourselfers, can now expect to succeed in molecular cloning on first attempt within a week. As few as one and a half more weeks are sufficient for designing and building altered gene functions with a reasonable chance of success. Longer times provide for trouble-shooting and also encourage more creativity and self-directed learning. Should more than five weeks or alternative projects be desired, other experimental suggestions are given at the end of this chapter. All protocols can be completed, or at least taken to a potential storage point, within half a day, adding further flexibility. Although bacterial cultures and some PCR reactions require overnight incubations, these incubations are performed unsupervised and their main requirement is starting them at the end of the day.

Space and major equipment requirements for 27 students were given in Chapter 3. The quantities of equipment, disposables, chemicals and molecular biologicals are given in Tables 2–6 in Appendices and are calculated to fit 27 students working for five weeks.

PROJECT OVERVIEW AND LEARNING OBJECTIVES

A central feature of synthetic biology is engineering the function of genes in a manner that is seen at the organismal level. One of the most dramatic and recent illustrations of this is to color bacteria by expressing a chromoprotein, the first step of this course. The aim of the remainder of the course is to up- and down-regulate the protein expression level in a rationally designed way. The main methods are BioBrick™ DNA cloning and

PCR mutagenesis. At the end of the lab course, students should be able to:

- understand basic principles in synthetic biology;
- perform experimental protocols central to synthetic biology;
- use the scientific method to generate hypotheses, design experiments to test them and analyze the results;
- document and present clearly their methods, results and conclusions.

THE LAB NOTEBOOK

Laboratory notebooks always have been, and still are (despite the advent of personal computers), essential for research. For this reason, we recommend that student lab books be assessed for a portion of student grades (see "The dreaded exam" at the end of this chapter). At a minimum, the lab book documents methods in sufficient detail that they can be repeated by you and others (a cornerstone of science), documents results so they can be analyzed (and hopefully published!) and documents stored samples so they can be used with confidence. Documentation should be clear enough that scientists in your field at your institution can understand it without you explaining it.

A proper lab notebook has numbered pages which cannot be removed. If there are no page numbers on your book, number each page now. All entries should be written in ink, not pencil, so they cannot be erased. On the outside cover, write your name, book number and laboratory. Inside the front cover, provide contact information in case the book is lost or stolen. Write

"Contents" at the top of the first two pages and then proceed to write up your experiments sequentially from front to back. Typed protocols can be pasted at the back. An important tip is to always write directly into your notebook and not to keep a second notepad on the side for drafts. By following this tip, potentially important information will not be lost and you will not lose time (and perhaps accuracy!) in copying it into your lab book. Any mistakes should be crossed out but remain readable. It is important that you do not leave any big open patches — cross out such patches before moving to the next page. Adding data in retrospect or erasing, removing or destroying data is prohibited. This can be important for fraud, patent or even Nobel Committee investigations!

In your lab notebook, write down everything you plan, do, notice and analyze throughout the day in the lab. Everyone writes their own lab notebooks in their own way, but there must be a structure to it. For every experiment include headings in the following order:

- Title (at the top of the page),
- Date (in unambiguous international YYYY-MM-DD format) in the left-hand margin,
- Aim or Hypothesis,
- Methods,
- Results,
- Conclusions.

Data, readouts, gel images, photos, *etc.* are to be properly attached in the lab notebook. Wherever possible, use original documentation (*e.g.* a machine readout with date and data, rather than hand copying out the data). Anything that is impossible to store in a lab book (*e.g.* due to size) should be dated and referred to clearly in the lab book. Writing thorough Results and Conclusions will

help you support or refute your hypothesis and develop new hypotheses. Forcing yourself to write down detailed conclusions often leads to ones that were not apparent when looking at all the data at once. Ideally start with discussing negative and positive controls: if either failed, your experiment is probably a wash! Then go over all other gel lanes, agar plates, *etc.*, one by one.

LAB SECTION 1. PREPARATION OF CHEMICAL SOLUTIONS AND AGAR PLATES

Protocol 1 (Chapter 6). In order to perform the experiments, all six chemical solutions and the plates listed in Protocol 1 are required. Schedule preparation of the six solutions on the first day and the agar plates on the second day because of the autoclaving delay (Table 7 in Appendices). To avoid several groups competing for the same chemical stocks, the lab teacher can specify different orders of preparation of solutions for different groups. Alternatively, if time is severely limited, teachers could prepare the solutions for the students beforehand. The same applies to LB antibiotic agar plates; teachers may supply LB plates for initial or all rounds of cloning. Some labs have a media facility that provides agar plates.

The importance of preparing solutions correctly cannot be over-emphasized. Trouble-shooting a failed experiment is hard enough without having to second guess whether or not the problem was due to an incorrectly made or contaminated solution. For this reason, it is crucial to document in your lab book the exact calculations used to make up your solutions. Otherwise how can you check if there was a mistake? Use common sense regarding accuracy of weights and volumes: for biological experiments, four significant figures are not required!

LAB SECTION 2. COLORING BACTERIA BY ADDING A PROMOTER TO A CHROMOPROTEIN GENE

Remember that **for protein expression, four DNA parts must be combined** (Fig. 5):

• A promoter, for initiation of transcription.
• A ribosome binding site (RBS), for initiation of translation.
• A protein coding sequence (CDS or ORF), for translation elongation and termination.
• A plasmid vector for stable maintenance and replication of the above three parts. (An alternative discussed in Chapter 7 is a chromosomal integration site.)

Fortunately for us, these four types of DNA parts have been standardized by synthetic biologists as BioBricks™ so they can be assembled easily (like Lego® blocks). The assembly process is plasmid DNA cloning using restriction endonucleases and DNA ligase, invented 40 years ago by Boyer and Cohen, but in a much simpler, standardized form (Knight, 2003) called **BioBrick™ assembly** (Fig. 23).

BioBrick™ plasmids are available free from a central distribution facility, the Registry of Standard Biological Parts.

The Registry of Standard Biological Parts

The iGEM Foundation handles the parts registry, which has existed since 2003: http://parts.igem.org/ Main_Page. They list and handle thousands of parts inserted in plasmids, almost all of which are BioBrick™ compatible. All constructs used in this lab

course can be ordered from the registry (Table 1 in Appendices). BioBricks™ have a helpful nomenclature: http://parts.igem.org/Help:Plasmid_backbones/Nomenclature.

Each part is defined by a code beginning with **BBa_** and the plasmid backbone flanking each part is coded as **pSB#X#**, where

- pSB = plasmid Synthetic Biology (*i.e.* a BioBrick™ vector);
- first number = origin of replication (*e.g.* 1 is high copy, 3 is low-medium copy);
- fourth letter = antibiotic resistance (*e.g.* C = chloramphenicol; this version is available for almost all inserts);
- second number = the generation of the vector.

Most commonly, parts are inserted in pSB1C3, a high copy plasmid vector. Sometimes vectors are called parts and sometimes not. The nomenclature does not refer to the particular BioBrick™ assembly standard used, but it is usually RFC10 and the web page for each part in the registry shows which standards the plasmid is compatible with. For information on the five assembly standards supported by the registry, see http://parts.igem.org/Help:Standards/Assembly.

For their first challenge, students will engineer protein expression by assembling the parts discussed above (promoter, RBS and CDS) into one destination plasmid (Fig. 24A) using BioBrick™ RFC10 assembly.
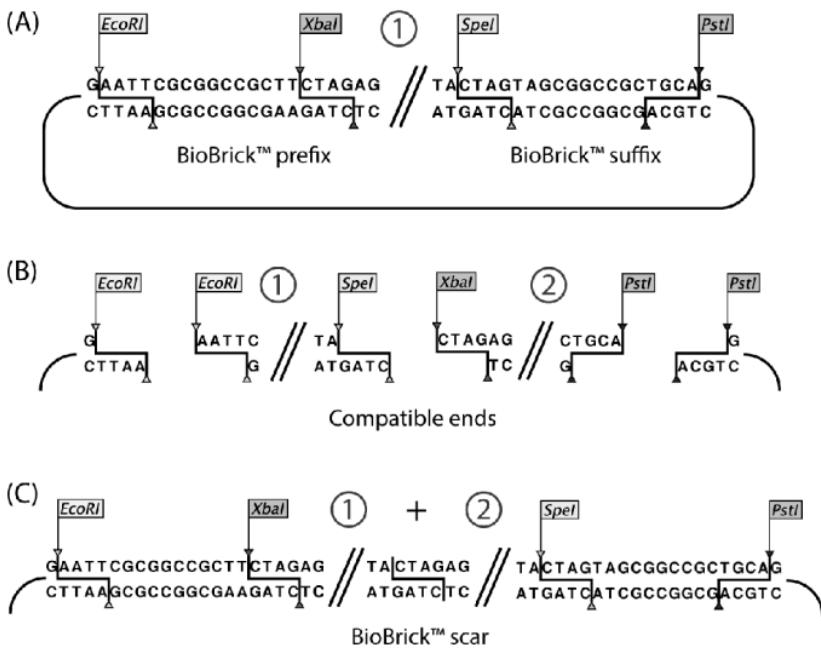
Fig. 23 The original and most commonly used BioBrick™ assembly standard, RFC10. (**A**) BioBrick™ plasmid containing part 1. The RFC10 standard uses the exact prefix and suffix sequences shown and only four restriction enzymes, EcoRI, XbaI, SpeI and PstI, that cleave under the same conditions. Their recognition sites must occur only once per plasmid at the flanking locations shown. For coding region parts starting with ATG, the 3'-terminal AG of the prefix sequence is omitted (http://parts.igem.org/Help:Prefix-Suffix). (**B**) Digestions forcing cloning of part 1 upstream of part 2 into a destination plasmid vector. Alternatively, part 1 could be cut as shown for part 2 and part 2 could be cut as shown for part 1 to force clone part 1 *downstream* of part 2 (not shown). (**C**) Ligation of the fragments in **B** utilizes the compatible sticky ends (overhangs) of XbaI and SpeI to create a "scar" which cannot be cut with either enzyme. The end result is a new BioBrick™ plasmid containing a larger part, labelled 1 + 2, flanked by the standard prefix and suffix. For simplicity, NotI restriction enzyme sites are not shown.
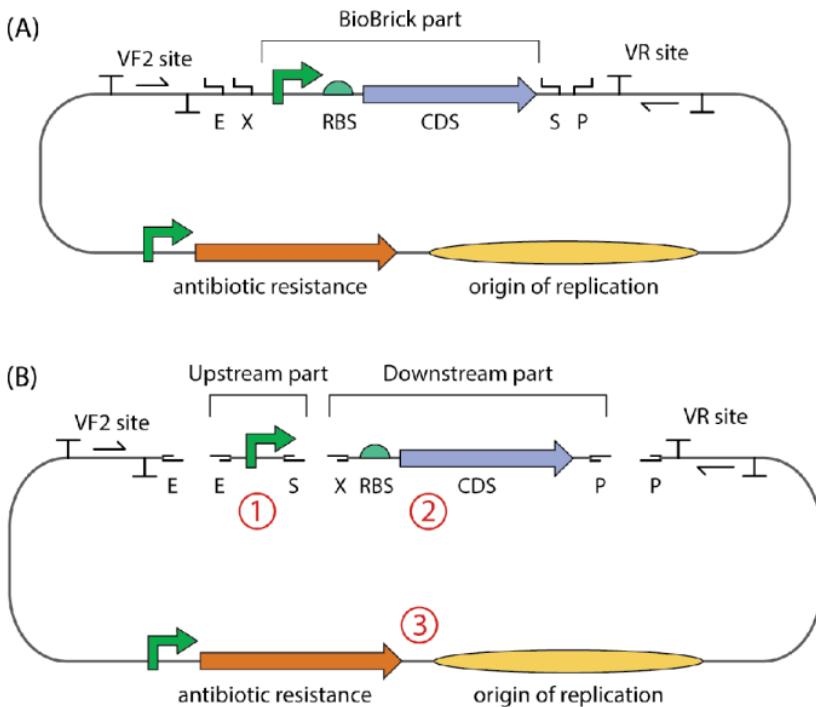
Fig. 24 (**A**) Illustration of a plasmid containing a promoter, RBS and coding sequence (CDS) of interest. (**B**) Construction of the plasmid in **A** from two BioBrick™ parts numbered 1 and 2 (according to the scheme in Fig. 23B) and a destination plasmid vector labelled 3. E, X, S and P represent EcoRI, XbaI, SpeI and PstI restriction sites.

To further simplify assembly, the RBS and chromoprotein CDS parts have already been combined into one part. Thus the chromoprotein-expressing plasmid only needs to be assembled from three different DNA restriction fragments (Fig. 24B):

1. promoter (upstream part),
2. RBS-chromoprotein CDS (downstream part),
3. plasmid vector (backbone part).

Successful *in vitro* assembly of the plasmid followed by transfer of the plasmid into bacteria (transformation) will start steady (constitutive) expression of the chromoprotein *in vivo*. This process will be directly visible as colorful bacterial colonies on the next day. We recommend that students are given different unknown chromoprotein genes as a starting point so they may practice the scientific method in determining which genes they have.

The plasmid construction begins not with the three restriction fragments themselves, but with three cell strains, each containing one of the three different DNA plasmids (top of Fig. 25):

1. Plasmid containing promoter insert BBa_J23110 bounded by BioBrick™ restriction sites (upstream part). It should be mentioned that this plasmid also contains a downstream red fluorescent protein (RFP) reporter CDS between the S and P sites; this is not shown in Fig. 25 because this CDS can be neglected using the standard 3A assembly method.
2. "Mystery" plasmid containing a RBS-chromoprotein CDS insert BBa_K10339## bounded by BioBrick™ restriction sites (downstream part; Table 1 in Appendices).
3. Medium-low copy plasmid pSB3K3 containing a reporter gene insert (RFP) bounded by BioBrick™ restriction sites (destination vector or backbone part). **A parallel cloning is also recommended** using a different destination vector, high copy plasmid pSB1K3, as comparing the color intensities produced from the two different vectors will aid in identification of the mystery chromoprotein gene.
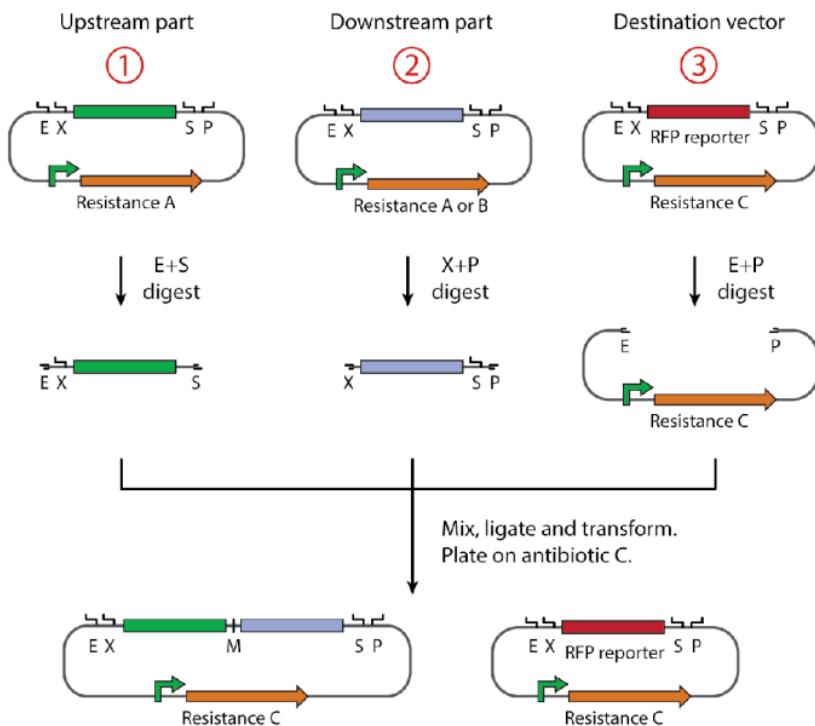
Fig. 25 BioBrick™ "3A assembly" standard. Although 3A is an abbreviation for 3 Antibiotics, only two different antibiotic resistances are required. This method is a second-generation BioBrick™ method designed to circumvent the need to gel purify restriction fragments. Here, the upstream part and the downstream part are digested out of their respective plasmids and ligated into a new (destination) vector plasmid encoding different antibiotic resistance. This resistance allows selection against re-ligations among fragments just from the two plasmids containing the upstream and downstream parts. Although selection against re-ligation of cut destination vector is not possible based on antibiotic resistance, such unwanted events are readily visualized on plates because the RFP reporter fragment is re-activated to give red colonies. The numbering and color schemes are those of Fig. 24. Part 1 for our course lab contains an RFP reporter CDS between the S and P sites that is not shown here; this reporter can be neglected when using the standard 3A assembly method.

Plasmid copy numbers available in the Registry

The plasmids used in this manual have either the medium-low copy origin P15A (pSB3X#; copy number of 10–12) or the high copy origin pMB1 (pSB1X#; copy number of 500–700). At the time of writing, there do not seem to be any functional low copy plasmids available from the Registry of Standard Biological parts. Although there are plasmids there based on the low copy origin pSC101 (pSB4X#), these have displayed anomalously high copy behavior in our university and in the hands of other iGEM teams.

The work flow is shown in Fig. 26.

Note that the promoter strain 1 has red colonies due to its RFP reporter, each mystery strain 2 has white colonies because it does not express its chromoprotein CDS, and the two alternative destination vector strains 3 have red colonies due to expression of the RFP reporter.

Protocol 2 (Chapter 6) is next, amplifying and purifying plasmids. Make up and inoculate four media solutions, each containing the appropriate antibiotic (Table 1 in Appendices) corresponding to the antibiotic resistance encoded by each of the four plasmids.

Antibiotic selection markers

When transforming and growing bacteria, it is important to select for only those cells that carry the desired plasmid. This is accomplished by incorporating an antibiotic resistance gene into the plasmid backbone and by growing in the presence of the antibiotic; only cells carrying the antibiotic resistance gene will be

protected and survive (positive selection). Resistance genes can work through different mechanisms, such as pumping the antibiotic out from the cells or enzymatic degradation of the antibiotic. Note that some antibiotic-degrading enzymes are secreted from the cells, meaning that with time even non-resistant cells can grow. This is mainly problematic with **ampicillin resistance** and can be observed on a plate when larger colonies of resistant bacteria become surrounded by smaller colonies of sensitive bacteria.
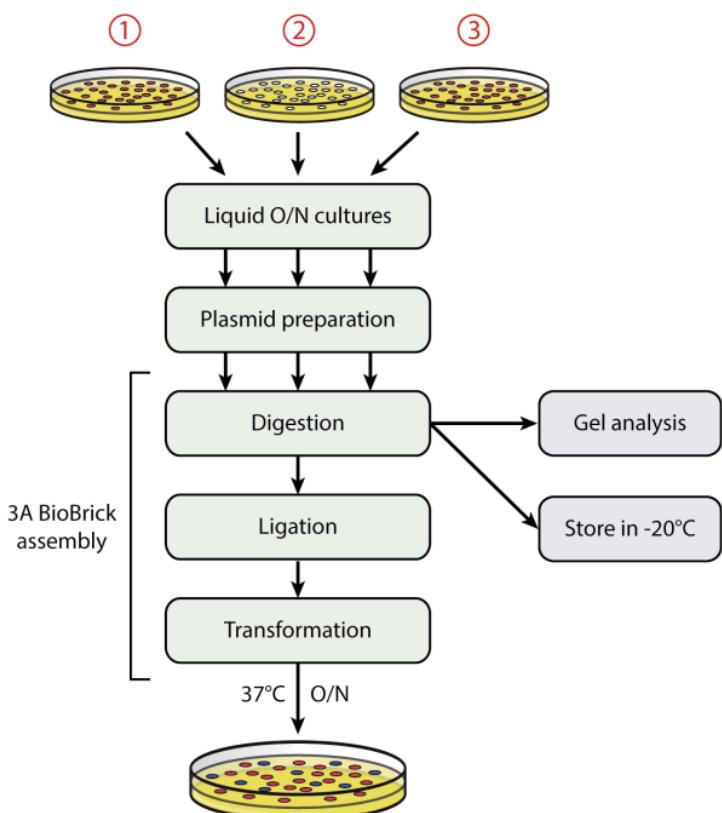
Fig. 26 Work flow for 3A assembly starting from three agar plates corresponding to parts 1, 2 and 3 of Fig. 25. For the destination vector part 3, trying two alternatives with different copy numbers is recommended.

By the next morning, all four cultures should have became very cloudy, *i.e.* grown to saturation. Now the four plasmid DNAs can be harvested using a plasmid prep kit according to the manufacturer's instructions. All four plasmid preps should give column eluates with high optical densities at 260 nm. Can you attribute any differences between yields to intrinsic features of the plasmids or procedural variables?

Plasmid miniprep kits

Kits from different manufacturers have different components and procedures. Therefore, always follow the manufacturer's instructions precisely. The first step is overnight culturing in 1–5 mL of medium. The cells are collected by centrifugation and resuspended in a buffer containing ribonuclease.

Next comes the addition of lysis buffer, a highly alkaline solution of the detergent, sodium dodecyl sulphate (SDS). The lysate is then neutralized and centrifuged to pellet cell debris, which includes cell wall, denatured proteins and chromosomal DNA. The supernatant, which is enriched for supercoiled plasmid DNA, is then loaded onto a silica column in the presence of a high salt concentration. The column is then washed to remove contaminants before elution of the plasmid.

Protocols 3–6 are next, generating the DNA fragments shown in Fig. 24B and assembling them by BioBrick™ 3A assembly (Fig. 25). Assuming two different destination vectors were prepared, four separate restriction diges-

tion incubations and two separate ligations will be performed.

Results will be analyzed over three days (Table 7 in Appendices). On the first day, digests are analyzed by gel electrophoresis. Then, in the morning of the third day, the cloning is analyzed by visualization of colonies on plates. Colonies harboring plasmids with a promoter upstream of a chromoprotein gene will have the color dictated by that particular chromoprotein. Does your choice of colored paper placed beneath the agar plate affect your sensitivity in detecting faintly colored colonies? Can you tell from the color of your colonies which chromoprotein gene you have? Note that chromoprotein color development may be slow due to the need for chemical maturation (Chapter 2). If some colonies on the plates are white after overnight incubation at 37°C, incubating them at 42°C for a couple of hours in the morning may speed up color development. Although this cloning experiment is not designed to generate white colonies, they usually occur anyway: what might they be? Document the results of each test and control plate: detailed descriptions may be sufficient, but feel free to use cell phone photography! A typical example is shown in Fig. 27.

Protocol 7 is next, amplifying and characterizing promising plasmids. This involves amplification/purification by re-streaking (Figs. 27 and 28), liquid culture, plasmid preparation and characterization by digestion/gel electrophoresis and DNA sequencing (Fig. 29).

The analytical digestion is best performed by excising the insert with EcoRI and PstI, the two enzymes used to cut the destination vector. This will

Fig. 27 The expression level of aeBlue chromoprotein varies with plasmid copy number. The copy numbers of the plasmid vectors used were medium-low (left) and high (right).
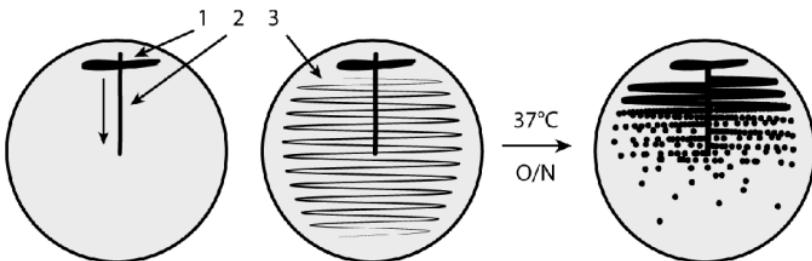
Fig. 28 Generating many single colonies by re-streaking out one colony. This three-step procedure is the alternative 1 detailed in Protocol 7.

test if the insert is the size expected for giving the bacterial color observed (although it does not rule out multimeric inserts) and if the ends of the insert and vector are still intact (ends are susceptible to exonuclease "nibbling" of one or a few nucleotides during cloning). Finally, if the digest gave the correct-sized fragments, the plasmid should be sent for DNA sequencing across the insert using the VF2 primer (Fig. 24A). The exact method of preparation of samples
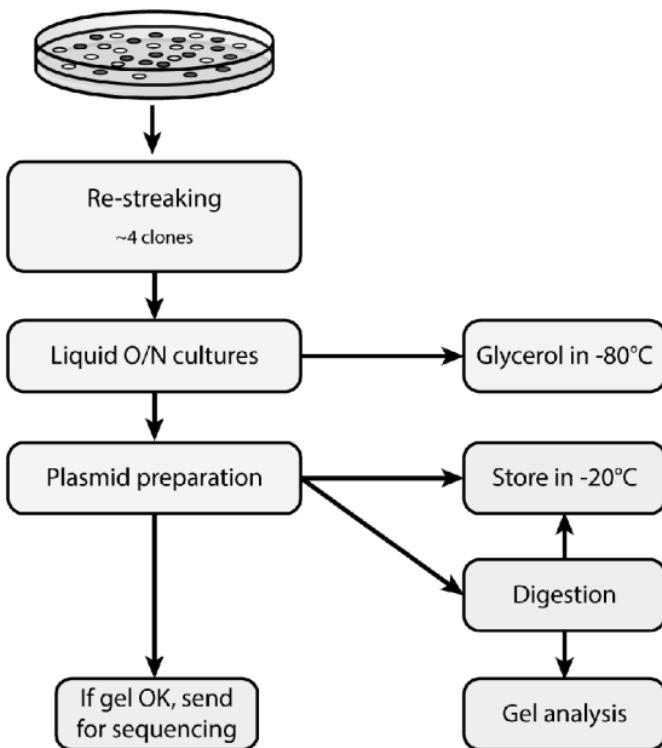
Fig. 29 Work flow for characterizing the colonies of a 3A-assembly transformation plate.

for sequencing varies according to the sequencing facility used.

Sequencing DNA

Upon submitting plasmid or PCR DNAs to a sequencing facility, most commonly Sanger sequencing is performed. In Sanger sequencing, a synthetic oligodeoxyribonucleotide primer is hybridized upstream of the region of interest and the primer extended on the template by a DNA polymerase.

Besides the four dNTPs, four dideoxyNTPs with specific fluorophores are also added to the reaction. The dideoxyNTP terminates the primer extension due to lack of a 3'OH group necessary for phosphodiester bond formation. The products of the primer extension are then separated through a column and at the end a laser excites the fluorescent label on the last nucleotide. A camera detects the color and this reveals the sequence of the template, one nucleotide at a time. Beyond Sanger sequencing, next-generation sequencing technologies enable much higher throughput and can be much more time and cost effective, depending on the type of project.

While waiting for DNA sequences, students may either:

 (i) finish Lab section 2 by performing colorimetric assays (see next paragraph) or

(ii) get a head start on Lab section 3 (see below) by designing and ordering PCR primers because primer synthesis and delivery takes a few days (Table 7 in Appendices).

Colorimetric Assays for Chromoprotein Expression

It is now time to practice an important element of synthetic biology, measurement of the activities of DNA parts. This assay is necessary to determine the effects of mutations that you will create for up- and down-regulating chromoprotein expression. Presently there is no accepted standard method for quantifying chromoprotein expression, so creativity is encouraged! Visual inspection of the

colors of the bacteria in solid and liquid cultures and in pellets provides a qualitative estimate of chromoprotein expression level, and this can be documented readily by color photography.

- But can you quantitate expression levels with numbers?
- Might a spectrophotometer be helpful?
- If so, what wavelength(s) should you use?
- If your chromoprotein absorbs light at OD_{600}, the wavelength typically used to monitor *E. coli* growth, how can you control for different extents of growth?
- What are appropriate controls to test this?
- What is your signal-to-noise ratio?
- If you have access to a flow cytometer, might that be helpful (see Figs. 40 and 41 in Chapter 7)?
- Might cell lysis be helpful?

Protocol 8 is for lysis with lysozyme.

- Can you achieve lysis efficiently with this lysozyme protocol?
- How can the extent of cell lysis be estimated easily?
- Once you develop an assay for measuring expression level, how reproducible is it?
- What are your standard deviations and standard errors of measurement?

In preparation for these colorimetric assays, **four overnight cultures are needed:**

1. Your chromoprotein-expressing strain (test).
2. Its precursor strain lacking the promoter (control for the test; may not be a true negative control because of possible cryptic promoter activity upstream of the cloning site).

3. The cloning strain lacking a plasmid (negative control).

4. The most strongly colored strain from your lab class (positive control for lysis).

When your sequencing results come back, both the computer-deduced sequence and the raw sequence data will be provided. Software programs are available to help with the analysis. Are all the nucleotide peaks clearly interpretable or is your "clone" actually a mixture? Are the sequences of the parts correct? These sequences can be downloaded from the Registry of Standard Biological parts (see text box above) by typing in the BBa-code for the plasmid name (*e.g.* BBa_K1033931 brings up the sequence of amilGFP; Table 1 in Appendices). Are the junctions, such as the scar sequence, correct? If there is a mutation in the insert, would it be predicted to decrease the fitness cost of the plasmid to the cell?

Fitness costs and genetic stability

The function of synthetic systems in bacteria is always limited by the "metabolic budget" of the cells. Expression of foreign proteins or production of metabolites will consume both energy and resources that otherwise could have been used for growth, so the expression of synthetic systems almost always confers a fitness cost, making the bacteria grow slower than they otherwise would have done. When using strong promoters or when expressing toxic genes, this fitness cost can get very high, leading to a strong selection pressure to lose the genes responsible for this cost. If any bacteria in a population get

loss-of-function mutations in any of the genes causing the fitness cost, they will have a growth advantage compared to the surrounding bacteria, and they will be rapidly enriched in the population. This is especially problematic when using high copy plasmids, since a plasmid present in 100 copies per cell also will have a 100-fold bigger chance to pick up loss-of-function mutations compared to a construct present in only one copy. Simple loss-of-function mutations are frame shifts and stop codons within coding sequences. Recombination between homologous sequences flanking an insert is favored if one part is reused several times in a construct, *e.g.* when using the same standard terminator both downstream and upstream of an insert. This is one of the reasons it is important for the synthetic biology community to create larger libraries of standard parts that lack homologies with each other.

LAB SECTION 3: RATIONAL ENGINEERING OF CHROMOPROTEIN EXPRESSION LEVEL

Synthetic biologists usually alter protein expression from a plasmid by changing:

1. the plasmid copy number, affecting the number of genes;
2. the promoter, affecting initiation of transcription;
3. translation initiation sequences;
4. codon bias of the protein coding sequence (CDS), affecting translation initiation and elongation.

Less commonly, synthetic biologists adjust gene expression by changing:

5. translation by targeting the mRNA with antisense RNA;
6. the stability of the mRNA;
7. the stability of the protein.

Expression can be regulated by changing:

8. the RNA polymerase;
9. transcription by adding an operator sequence for binding a repressor protein that is inducible with a small molecule.

Change 1 has already been tested above by all groups, so now the groups will test each of Changes 2–5. Each group will pick one of these Changes 2–5 to pursue and attempt both up- and down-regulation of chromoprotein expression (except for Change 5, where up-regulation cannot easily be designed). If there are more than four groups, necessitating that two groups pursue the same change, they should test different hypotheses (*e.g.* for Change 3, up- and down-regulation could be achieved by one group through RBS mutations, while the other group could achieve up- and down-regulation by mutating the adjacent mRNA sequences to alter pairing with the RBS). From this point on, the designing and testing of specific hypotheses is driven by the creativity of the students (like iGEM), rather than teacher driven. So rather than providing detailed experimental design, we provide a list of questions and additional general experimental protocols helpful for answering these questions experimentally. Any mutations up to 80 straight base pairs in length are compatible with the mutagenesis method provided. So is randomization of several bases to create libraries.

Changes 2–5

- Using the **background given in Chapter 2** and any other information available in textbooks, scientific papers or on the Internet, can you design specific mutations to effect your desired changes in expression?

- First contemplate the plasmid you start with. If you made both high and medium-low copy number plasmids expressing your chromoprotein, are one or both suitable for testing up- or down-regulation, or would it be better to start with a plasmid(s) of another group? We picked two of the chromoprotein genes partly because they had not been codon optimized, thus making them ideal for codon bias experiments.

Change 2, promoter

- How strong is your promoter sequence?
- How does it compare with other promoters in the Registry of Standard Biological parts?
- What are the relative importances of the −35 box, the −10 box and the spacing between them?
- Can you activate transcription with a transcription enhancer sequence?

Change 3, translation initiation

- How strong is your RBS sequence and how should you change it?
- Is your RBS likely inhibited by base pairing with adjacent sequences in the mRNA?
- Can you change translation initiation by mutating sequences adjacent to the RBS in the mRNA?
- Can you activate translation with an epsilon translation enhancer sequence?
- Should the translation start codon be mutated?

Change 4, codon bias

- Has the CDS of your chromoprotein been codon optimized for *E. coli* (Table 1 in Appendices)? If not, it is probably better suited for codon bias experiments.
- Is the codon usage significantly different from that of highly expressed genes in *E. coli* (Fig. 9)?
- Are there any rare codons at the start of the CDS that might slow ribosome clearance from the initiation region or destabilize base pairing with the RBS?
- How big is the footprint of the ribosome on the mRNA?
- Are there rare codons further downstream?
- Should all the codons in the region you change be substituted with the most optimal codons?

Change 5, antisense

- What region of the mRNA would you target with antisense RNA?
- How long would you make the antisense sequence?
- How might this affect toxicity?
- What are the pros and cons of encoding the antisense RNA on the same plasmid versus a separate one?
- If a separate plasmid is used for the antisense RNA (top left of Fig. 50 in Chapter 8), is it compatible with the chromoprotein plasmid? For more information and examples of antisense results, see Fig. 36.
- Computational secondary structural modeling is useful for Changes 3 and 5 (mfold Web Server)

Protocols 9 and 10. The work flow for PCR mutagenesis and analysis (Fig. 30) differs from previous work flows in that it incorporates PCR (Fig. 31A).

The version of PCR we will use now is shown in Figs. 32 and 33A.

After the PCR reaction, remaining plasmid DNA is degraded by DpnI, a restriction endonuclease that cuts a specific methylated DNA sequence (Fig. 33B).
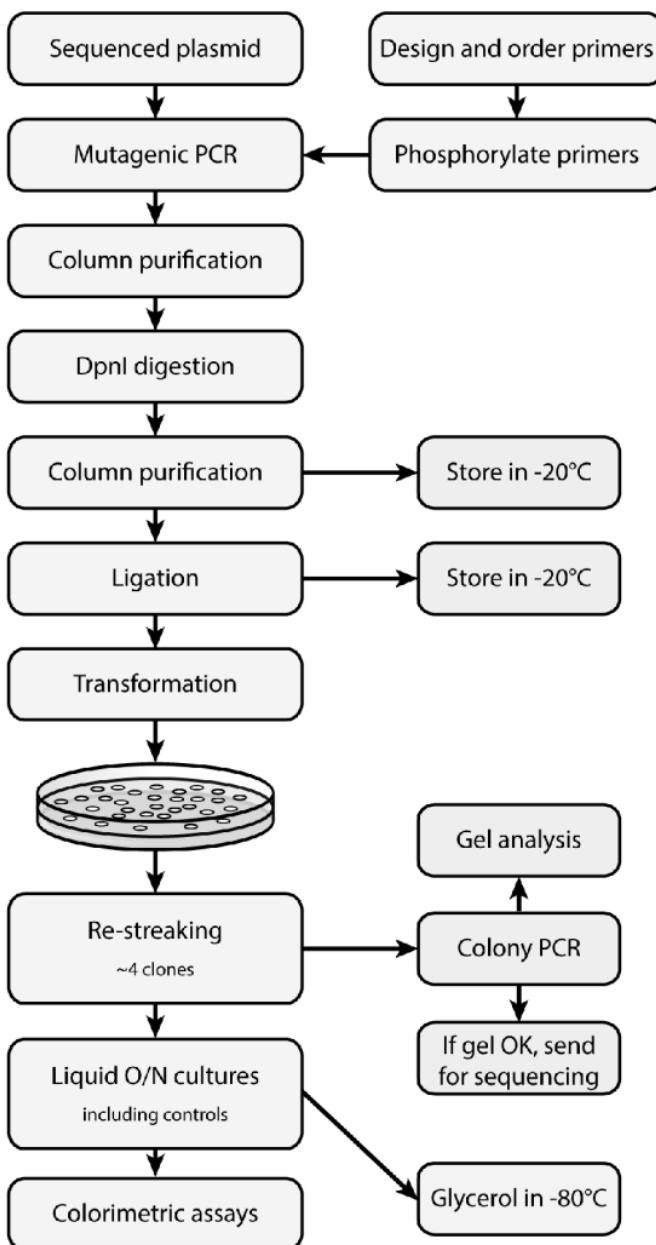
Fig. 30 Work flow for PCR mutagenesis and analysis.

If the transformation platings from PCR mutagenesis look promising, proceed to re-streaking and **Protocol 11**, colony PCR, to amplify your DNA for sequencing (Fig. 34).
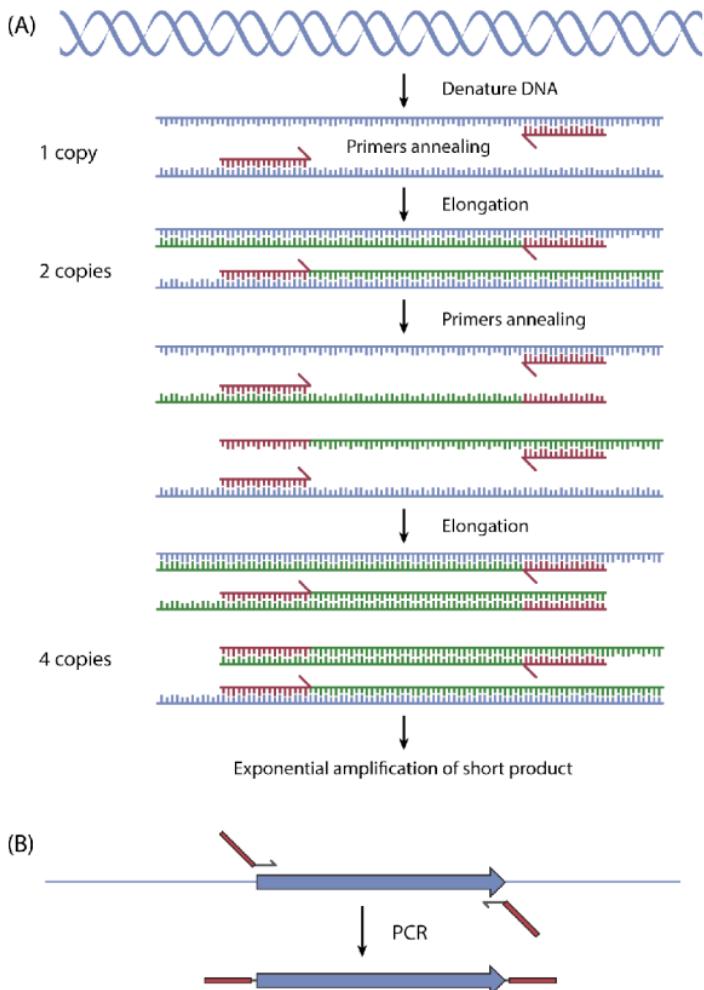
Fig. 31 The polymerase chain reaction (PCR). PCR is an *in vitro* technique for amplifying short segments of DNA. (**A**) The reaction starts with melting at high temperature of the double-stranded template DNA (blue) into single-stranded DNA. This is followed by a lower temperature step where two synthetic oligo primers (red) can bind specifically (anneal) to their target sequences. The third step is elongation, where a thermostable DNA polymerase synthesizes new strands of DNA (green) using the original template strands as templates. The DNA is then melted again and the cycle repeats. Every cycle the amount of the short product is doubled, leading to an exponential growth of product. (**B**) PCR can be used to modify easily any DNA sequence. The extra bases at the 5' ends of the primers (single-stranded "overhangs" in red) are added to the ends of the final amplified sequence.
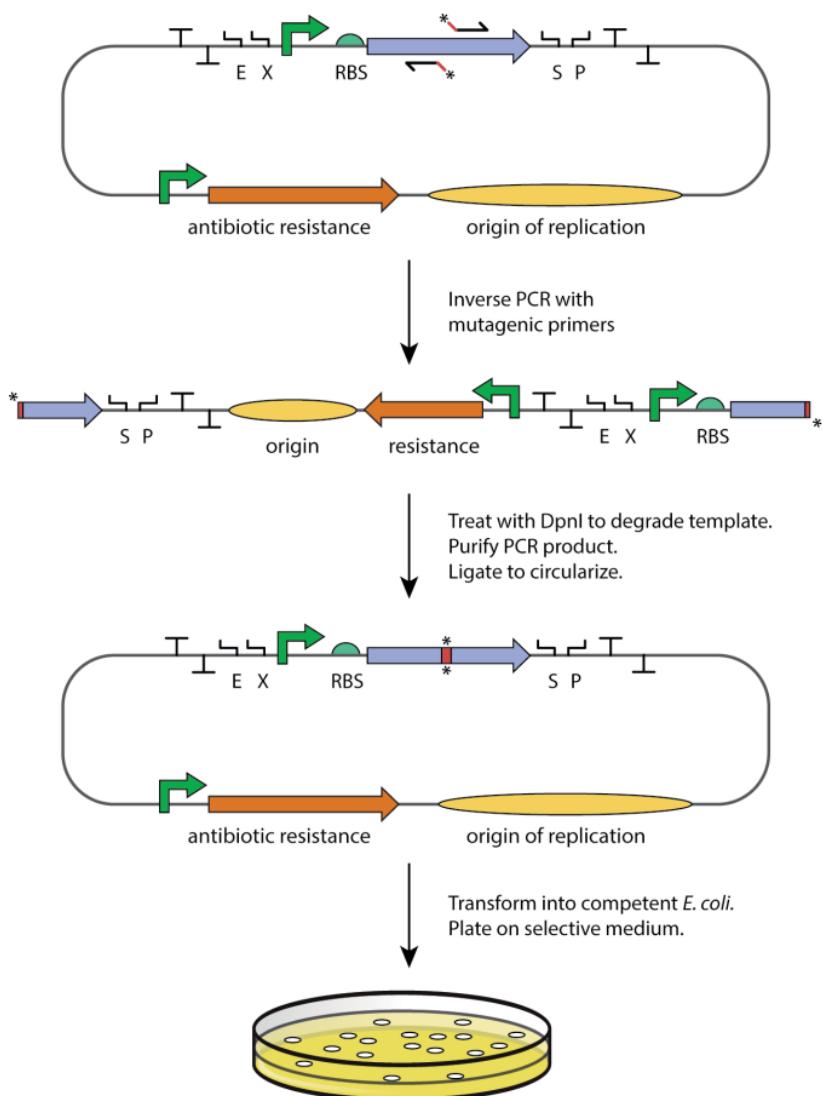
Fig. 32 Inverse PCR mutagenesis. The phosphates introduced by phosphorylation of the purchased synthetic oligodeoxyribonucleotides are marked with asterisks. The new sequences created (red) can be up to 80 straight bps and are derived from both primer overhangs.

(A)

(B)
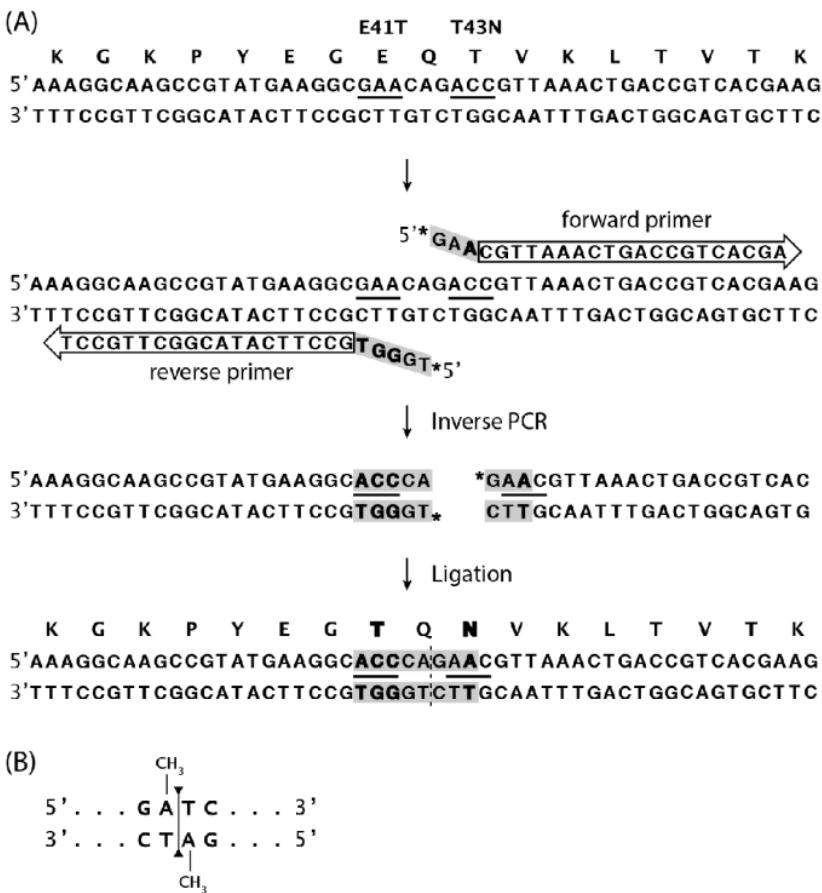
Fig. 33(A) Primer design for inverse PCR mutagenesis changing two amino acids. (B) DpnI cleaves the specific methylated sequence shown at the sites indicated with triangles. The methylation occurs *in vivo*, so plasmids are cleaved whereas PCR products are unmethylated and thus resistant to cleavage.

While waiting for sequencing results, use your colorimetric assays to quantitate expression to test your hypotheses concerning changing expression levels. Alternatively, if any prior BioBrick™ cloning or PCR mutagenesis experiments failed, they can be repeated or trouble-shooted.
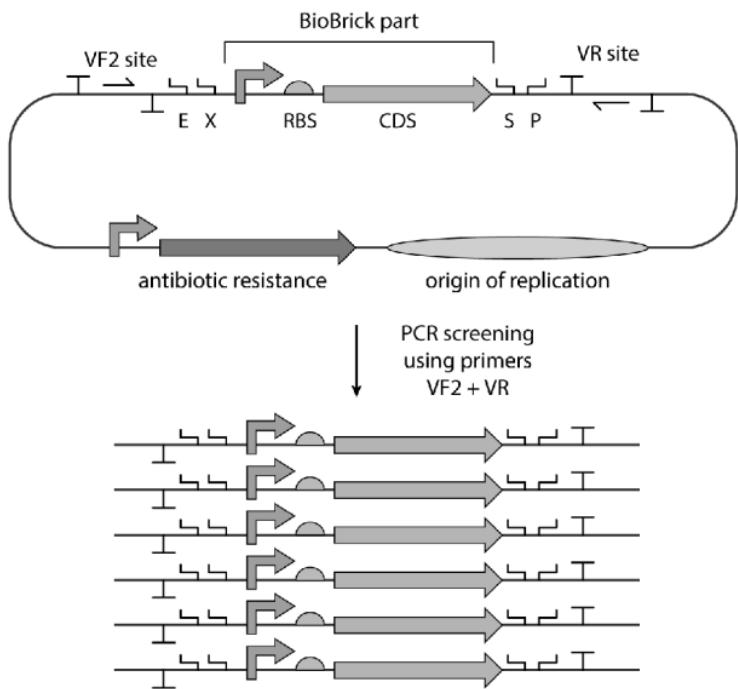
Fig. 34 Colony PCR enables amplification of DNA without purification from cells.

Suggestions for trouble-shooting failed experiments

- *Liquid culture of chromoprotein-expressing strain*
  - ○ Color is absent or incorrect
    - ▪ Not enough oxygen supply
    - ▪ Contaminative growth
  - ○ Color is weak or varies between preps
    - ▪ Not enough oxygen supply
    - ▪ Mutation(s) were selected mid-way during growth due to high fitness cost of very high expression of chromoprotein
      - ☐ Decrease expression level or temperature

- *PCR*
  - Multiple different products or incorrectly-sized product
    - Annealing temperature is too low. Note that the recommended annealing temperature is above or equal to T_m for Phusion® pol, but below T_m for Taq pol.
    - Mis-priming and extension occurred during initial denaturation step
      - Add the DNA polymerase to the hot wall of the tube *after* reaction mix reaches the denaturation temperature, then mix (without removal of the tube from PCR machine)
    - Too many reaction cycles
    - DNA contaminants
      - Purer template needed
    - Primers are non-specific
      - Order new (longer?) primers
      - Extract the correct product from the gel to purify from other products
      - Order two more primers for nested PCR
      - If there are extra bands below ~1000 bp in PCR mutagenesis, continue anyway according to the procedure. The origin of replication is incomplete in these small products, so they will not replicate *in vivo*
  - Product is absent or yield is low
    - Too little template
    - Too much template
    - Denaturing time is too short
    - Annealing temperature is too high
    - Too few reaction cycles

- Re-check T_m calculations for the primers
- Poor primer design
  □ Order new primers or add DMSO
- A component was left out of the PCR reaction

- *Ligation and transformation steps in PCR mutagenesis*
  ○ Few or no colonies after transformation
    - Transformation efficiency is too low
      □ Remake competent cells
    - Transformation used too much DNA, which is toxic to the cells
    - Non-functional or incorrect plasmid
      □ Prior steps failed
    - The new mutant is toxic to the cell
      □ Use a medium-low copy number plasmid
    - Add PEG to ligation
  ○ Too many colonies after transformation
    - The antibiotic in the plates has degraded
    - The DpnI treatment of the PCR template was inefficient
      □ Sequence several colonies
      □ Perform colony PCR screening if the mutation is expected to produce a difference in size measurable on an agarose gel
  ○ Deleted nucleotides at the blunt-end ligation site
    - Phusion® pol has 5′ exonuclease activity if incubation is too long
  ○ Extra nucleotides at the blunt-end ligation site
    - Taq pol was used by mistake. This pol adds on an extra adenine to the 3′ end

Our five-week course provided sufficient time for a second round of PCR mutagenesis. This can be performed directly on bacterial colonies, obviating another

plasmid prep. Aims in this second mutagenesis round included:

- Second-generation mutational design.
- Alteration of cell color by placing two different chromo-proteins in the same operon. Construction by 3A assembly requires another destination vector (pSB1A3 resistant to amp; Table 1 in Appendices) with different antibiotic resistance to the upstream part (promoter-chromoprotein gene resistant to kan) and downstream part (chromoprotein gene resistant to chloramp).
- Alteration of the color of the chromoprotein itself (Bulina *et al.*, 2002; Fig. 35).
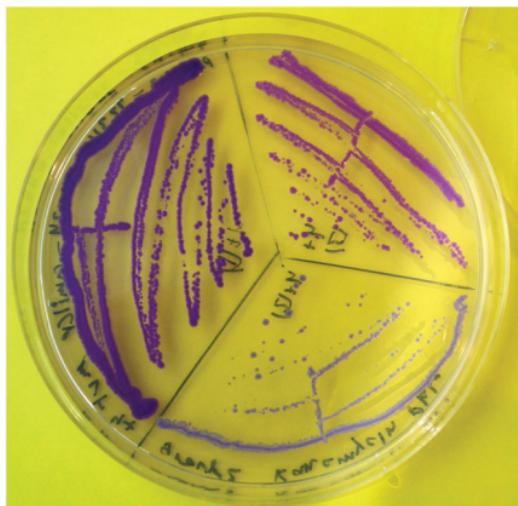
Fig. 35 Changing the color of amilCP chromoprotein. Amino acid C61 of amilCP (equivalent to C64 of gfasCP in Fig. 4 of Alieva *et al.*, 2008) was randomized by inverse PCR mutagenesis and colonies were screened by eye for colors different from amilCP. Three such colonies were re-streaked to give the agar plate in the photograph. PCR of individual colonies followed by DNA sequencing revealed the genotypes responsible for the phenotypes: C61F (left), C61G (right) and C61P (bottom). Single-letter abbreviations of the amino acids are given in Fig. 9. Experiment performed by Uppsala University students A. Berglund, J. Björkesten and V. Lindfors.
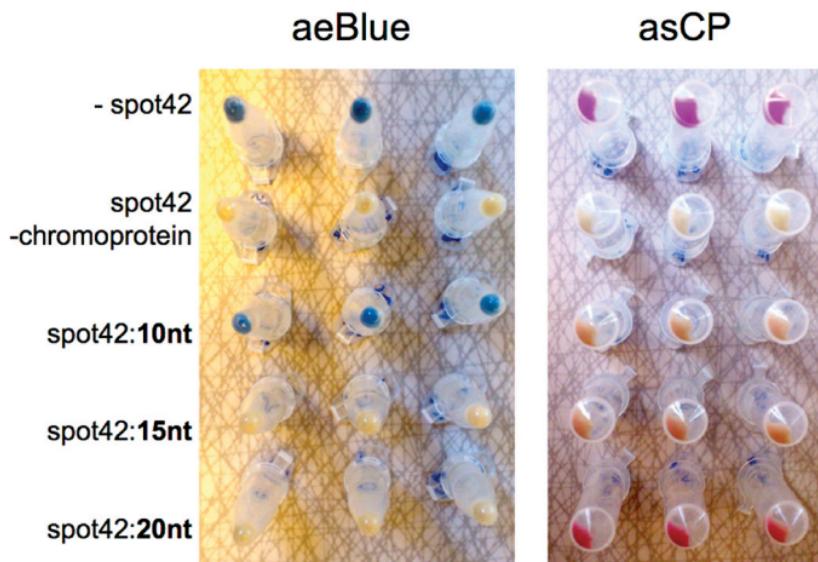
Fig. 36 Fine tuning of chromoprotein expression using sRNAs. Sequences complementary to the very first few codons of chromoproteins aeBlue and asCP (*i.e.* 10, 15 and 20 nts complementary to AUG…) were inserted by inverse PCR mutagenesis into the artificial sRNA based on spot42 (BBa_K864440 in Table 1 in Appendices; see also Figs. 11A and 50) at the site shown as a randomized region in Fig. 1b right of Sharma *et al.* (2011). Cells transformed with the combinations of chromoprotein-expressing plasmids (in vector pSB3K3) and spot42 plasmids indicated in the figure were amplified and pelleted in triplicate to give the results shown. The variation in inhibition of chromoprotein expression with length of complementary sequence differed significantly for the two different chromoproteins. Experiment performed by Uppsala University students P. Enström, A. Gynnå, M. Rahmani and N. Sandberg.

Another example of the class's experimental results is shown in Fig. 36 (see Cover).

LAB SECTION 4. OTHER EXPERIMENTS

Unlike BioBrick™ parts, most source DNAs lack conven-ient restriction sites for cloning. Although flanking BioBrick™ sites can be introduced readily by PCR for

cloning (Fig. 37), this strategy is problematic if there are "illegal" BioBrick™ sites internally.

Protocol 12, Gibson assembly, circumvents any problems due to unavailable or inconvenient restriction sites because it creates sticky ends without using restriction enzymes (Fig. 38).

Though Gibson assembly is often the method of choice for fusing two DNAs together, it was unnecessary for our lab course because course parts were designed to have compatible restriction sites. Nevertheless, we detail Gibson assembly in this manual because it, like BioBrick™ cloning, is a core technology of synthetic biology. It could be readily incorporated into future lab courses for putting any two parts together, such as placing two different chromoproteins in the same operon.

The main drawback of Gibson assembly is that it requires PCR, which has a much higher mutation rate than DNA amplification *in vivo*. Also, annealing of some ends can be inefficient, perhaps due to secondary structure formation at 50°C. This latter problem can usually be circumvented by overlap extension PCR (see Protocol 12 and Fig. 39).

Another important and flexible technology for joining DNA parts in synthetic biology is **recombination** *in vivo*. Like Gibson assembly, recombination methods such as Lambda Red recombineering also operate without restriction endonucleases. And recombination is an essential technique if genes are to be knocked out or inserted into the chromosome. Recombination is covered in Chapter 7 because it is probably more suitable for researchers and iGEM teams than as an initial classroom teaching tool.
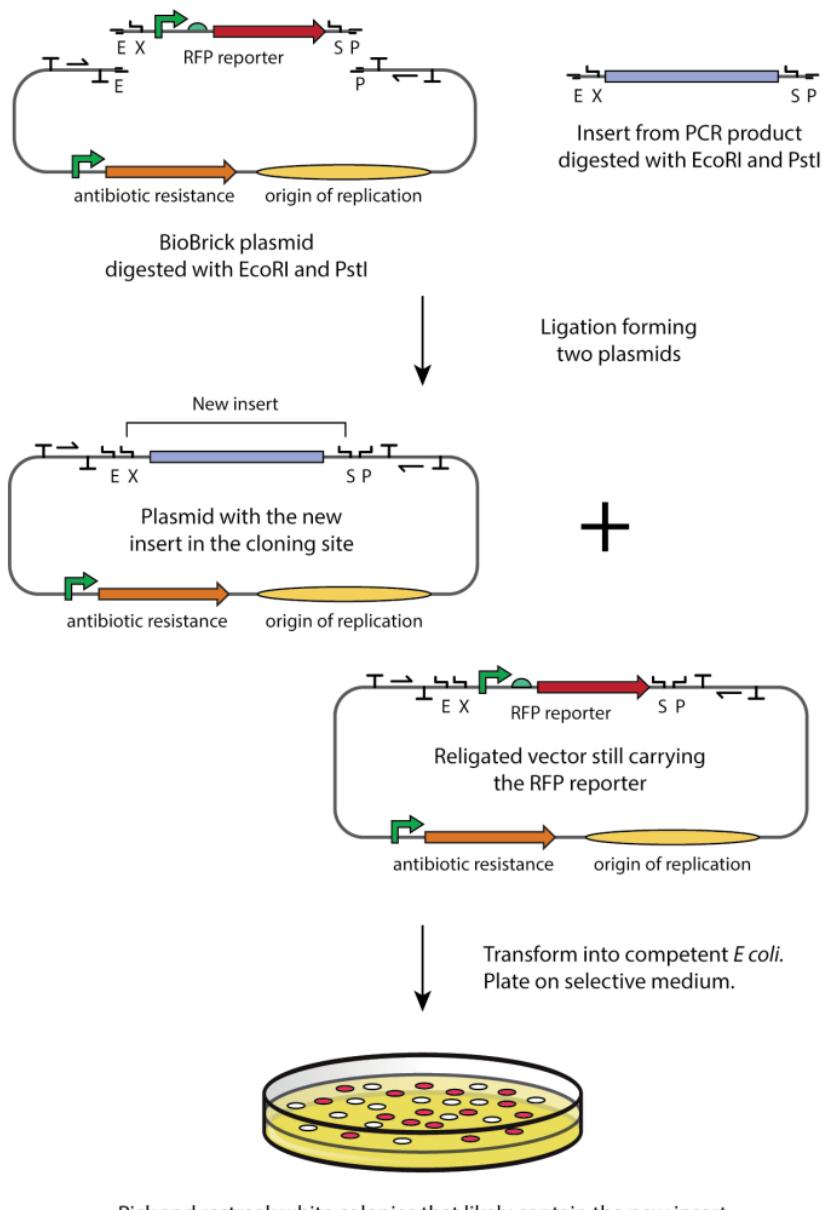
Fig. 37 Converting a non-BioBrick™ gene (blue) into a BioBrick™ part (middle). The first step is PCR using appropriate flanking primers (Fig. 31B). Note that primers should be designed with extra bases 5′ of the restriction sites to guarantee subsequent cutting. The second step is cloning using standard BioBrick™ restriction enonucleases and vector. This strategy is problematic if there are illegal BioBrick™ sites within the gene.
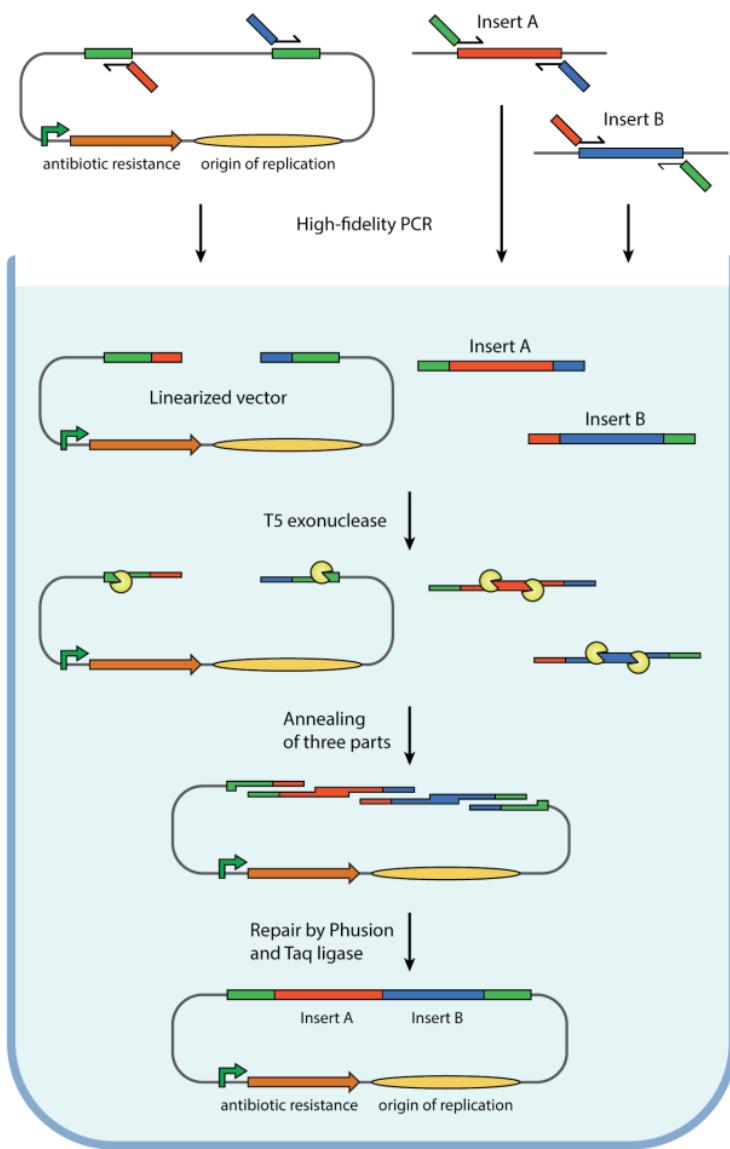
Fig. 38 Gibson assembly. This technique ligates one or more PCR fragments to a vector in one reaction mixture without restriction enzymes. First, PCR fragments are generated with primers that add overlapping sequences. Second, T5 exonuclease digests the 5′ ends, creating sticky ends that anneal. Third, Phusion®HF DNA polymerase repairs the single-stranded regions. Last, Taq DNA ligase forms phosphodiester bonds between the DNA sequences to seal the plasmid.
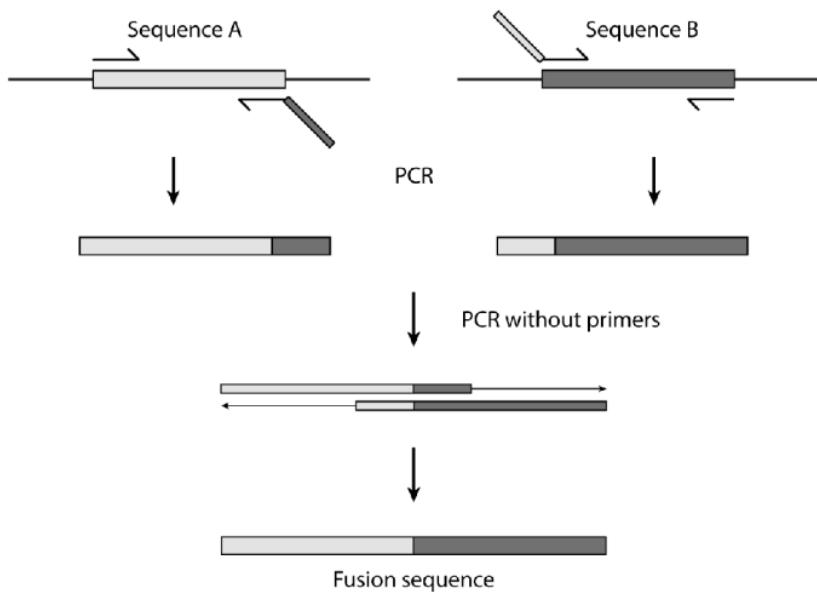
Fig. 39 Overlap extension PCR. This method is an alternative to Gibson assembly for joining two fragments without restriction enzymes.

BioBuilder

BioBuilder is a short practical course in synthetic biology for high school or early university levels, is based upon prize-winning iGEM projects and is available online: http://www.biobuilder.org/activities/

Detailed information is provided for teachers on how to set up the labs and computer simulation, and detailed protocols are provided for the students. We summarize the practicals briefly as follows:

1. *Eau that smell*

http://openwetware.org/wiki/BioBuilding:_Synthetic_ Biology_for_Students:_Lab_1

This lab is based on MIT's 2006 iGEM project. It uses banana odor as a readout of the growth phase of *E. coli.*

Three strains of *E. coli* are provided that have different expression patterns of ATF1 protein. This enzyme converts isoamyl alcohol to isoamyl acetate, the product responsible for banana's distinctive smell. Each strain expresses ATF1 from a different plasmid. One plasmid has a promoter that expresses the enzyme at stationary phase (BBa_J45250). The second plasmid has a different promoter (BBa_J45200) and the third plasmid has a genetic inverter (BBa_J45990), both of which switch expression to log phase. Students measure O.D.s and smell intensities from the three strains through lag, log and stationary phases. Duration: 3–5 days.

2. *iTune device*

http://openwetware.org/wiki/BioBuilding:_Synthetic_ Biology_for_Students:_Lab_2

This lab uses beta-galactosidase enzyme activity as a readout of expression levels of nine different strains of *E. coli*. The nine strains in the provided kit each harbor a plasmid encoding beta-galactosidase downstream of a different combination of weak, medium and strong promoters with weak, medium and strong RBSs. Duration: 3–4 days.

3. *Picture this*

http://openwetware.org/wiki/BioBuilding:_Synthetic_ Biology_for_Students:_Lab_3

This computer simulation is based on the 2004 iGEM project at U. Texas, Austin. It starts with computer modeling of the different regulatory parameters in a signal transduction pathway activated by light. A photograph

on a bacterial lawn can be produced in collaboration with the authors responsible for this lab. Duration: 3–4 days.

4. *What a colorful world*

http://openwetware.org/wiki/BioBuilding:_Synthetic_ Biology_for_Students:_Lab_4

This lab is based on Cambridge U's 2009 iGEM project. Two different plasmid-encoded operons for color production are transformed into two different strains of *E. coli* to compare the effect of chassis on expression. The operons encode enzyme pathways that make small molecule pigments (BBa_K274002 and BBa_K274003). The lab includes making competent cells through a simple version of the $CaCl_2$ technique. Duration: 2–3 days.

THE "DREADED" EXAM

Though everyone complains about exams, quality examinations are not only necessary to document mastery of a subject, they are also appreciated very much by students. But how best to examine the practical portion of a synthetic biology course? Whatever the assessment form takes, we believe such a big part of a course deserves a big part of the grades. But it can be hard to give individual grades for lab sections, especially when students work in groups. What worked well for us, at least according to anonymous course evaluations from the students, was the following:

• The student's degree of involvement in the lab was graded.
• Lab books were submitted to teachers for feedback after one week, then re-submitted for grading after completion

of the experiments. For guidelines on lab book preparation, see "The lab notebook" earlier in this chapter. A potential alternative suggestion would be to write up a report on the lab in the format of a scientific paper.

• Each group gave an oral powerpoint presentation where every group member had to participate equally.

The oral presentations were given to the whole class so all groups could learn about all of the experiments performed and everyone had the opportunity to ask questions. Presentation day was a major highlight of the whole synthetic biology course.

Presenting lab work orally using slides

• The temporal structure of the presentation should be:
  ○ Title slide. Include group member names;
  ○ Outline slide;
  ○ Introduction;
  ○ Aims/hypotheses;
  ○ Methods. Methods that were identical for all groups should only be mentioned briefly;
  ○ Results;
  ○ Conclusions/summary slide. Inconclusive results merit plenty of discussion, including potential future work.
• Keep to the time limit (25 min plus 5 min for questions worked well). The duration should be checked in a rehearsal.
• About one slide per minute is a good rule of thumb.
• Less important material should be placed at the end of the presentation so, if the group is running

out of time, it can be omitted without destroying the take-home messages of the talk.

- All slides are to be on white background with dark (black or dark colored) text using Arial or Helvetica fonts.
- Every slide should have a title describing its contents.
- One, maybe two, figures on each slide.
- Graphs should have clearly labeled axes, and the axes should be mentioned when discussing the graph.
- Every slide should have some bulleted text, but this should be kept to a minimum.
- The presentation should never be memorized word for word or read from notes.
- Speakers should be engaged with the audience and should encourage pertinent interruptions, as long as they are not too long!
- Questions should be answered briefly to leave time for other questions. It is O.K. to say that you do not know!

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6

Protocols

INTRODUCTION

Protocols are listed in order of use and contain all the methods necessary for the students. The text at the beginning of some protocols is written with both students and teachers in mind. The calculations for some of the solutions are left for the students because students preferred this more realistic training experience.

Before beginning lab work, know the safety procedures concerning fires, chemicals, biologicals and equipment detailed in Chapter 4. Prior to and after work each day, clean your lab bench with ethanol to try to ensure a sterile environment.

Read through the entire protocol before starting the experiment to prevent delays and to plan on minimizing the time that key reagents are removed from storage.

When removing enzymes from storage, always keep them chilled on ice and not for longer than necessary, otherwise they may lose activity. Remember to always add the enzyme last to a fully mixed solution, then mix again thoroughly. This is because enzymes are sensitive to inactivation by pH and ionic conditions that deviate from their storage and reaction buffers.

Storage of biologicals at low temperature

Storage at low temperature is crucial for the reuse over long periods of time of biologicals such as cells, protein enzymes and nucleic acids. But cells and protein enzymes in particular can be killed easily if cooled and/or stored in the incorrect manner. Rules differ depending on the individual biological, and there are many standard variables to consider, such as:

- temperatures of $-80°C$, $-20°C$ or $4°C$;
- water: dry (by lyophilization), wet or frozen;
- buffer: add glycerol and/or reducing agent, *etc.*;
- freezing rate: snap versus slow;
- aliquots: if only one cycle of freezing and thawing is recommended;
- history: has the sample been frozen before and, if so, how many times?

So it is important to read about storage in our protocols and in the manufacturer's instructions and/ or to ask the teacher if you are unsure. In general, frequently used enzymes and reagents are stored in a $-20°C$ freezer. Enzyme solutions in this category typically contain glycerol to prevent freezing.

However, if you wish to store samples for a longer period of time or they are less stable, a freezer with a temperature of −70°C to −80°C can be key. Examples of −80°C samples are competent cells, glycerol stocks of cultures, and enzymes from large purifications. When going into −80°C boxes it is important not to thaw other samples; *i.e.* only open the freezer or the box briefly, and make sure the door/lid is closed properly.

PROTOCOL 1. PREPARATION OF SOLUTIONS AND AGAR PLATES

Introduction

This protocol details the preparation of every solution necessary to perform the lab course. A pH meter is not required (except for Protocol 8, where the lab teachers should perform the steps with the pH meter). Final pHs should be verified with narrow-range pH paper. ddH_2O is either doubly distilled or of similar high purity. Solids should always be dissolved in a smaller volume of ddH_2O than the final calculated volume. For some solutions, autoclaving may be required to completely dissolve the solids (*e.g.* agar).

Useful equations

moles = mass/formula weight,   *where mass is in g*
=> mass = mol × FW

molarity = moles/volume,   *where molarity is in*
=> mol = M × L   *M and volume is in L*

1% = 1 g/100 mL,   *where % is assumed to be m/v, unless v/v or m/m is stated*

DNA concentration,   *where OD_{260} = optical*
= OD_{260} × 50 ng/μL   *density at 260 nm*

Notes:

0.9% NaCl (Also Known as Isotonic Solution), 10 mL

Introduction

This solution is used for suspending cells to avoid early lysis by osmosis. Some laboratories use phosphate buffered saline (PBS) instead.

Components

- NaCl;
- ddH_2O.

Procedure

1. Check that the balance is calibrated
2. Calculate how much you need to weigh out of the compound for making a 10 mL solution.
3. Add the salt to a glass bottle and add water to dissolve.
4. Make up to the final volume of 10 mL.

Notes:

50% (v/v) Glycerol, 50 mL

Follow the autoclaving instructions from the lab teachers.

Introduction

This solution is to be used for making cell glycerol stocks of important bacterial strains.

Components

- Glycerol stock;
- ddH$_2$O.

Procedure

1. Check which percentage glycerol is in the stock.
2. Calculate how much volume you need of glycerol and how much water you need to add to reach a final volume of 50 mL.
3. Measure the glycerol in a measuring cylinder.
4. Add to a glass bottle and add water to make a 50% glycerol solution.
5. Autoclave for 20 min.

Notes:

1 M CaCl$_2$

Follow the autoclaving instructions from the lab teachers.

Introduction

CaCl$_2$ is used in a less concentrated form for making competent *E. coli* cells. Upon exposure to CaCl$_2$, the cell wall becomes fragile and at 42°C *E. coli* takes up foreign plasmids efficiently.

Components

- CaCl$_2$;
- ddH$_2$O.

Procedure

1. Look up the molecular weight of CaCl$_2$.
2. Calculate how much CaCl$_2$ you need to weigh to give 10 mL of 1 M CaCl$_2$ solution.
3. Weigh out the powder and add it to 8 ml water.
4. Stir and shake until it dissolves.
5. Make up to 10 mL final volume.
6. Autoclave for 20 min.

Notes:

10x TBE Buffer

Na_2EDTA or Na_4EDTA can be used since their different effects on pH can be neglected for this buffer. The concentration in the gel and running buffer should always be 1x TBE.

Introduction

TBE stands for the three components Tris, boric acid and EDTA. EDTA chelates Mg^{2+}, both inhibiting enzymatic degradation of nucleic acid and giving sharper bands on gels. There is no risk of contaminative growth in 10x stock solution, but over time the salt may precipitate, requiring preparation of fresh buffer.

Components

- Tris base;
- boric acid;
- EDTA;
- ddH_2O.

Procedure

1. Dissolve the following reagents in 400 ml ddH_2O using a magnetic stirrer:
   Tris to a final concentration of 0.89 M;
   boric acid to a final concentration of 0.89 M.
2. Dissolve Na_2EDTA or Na_4EDTA to a final concentration of 25 mM.
3. Adjust the volume to 0.5 L. The pH will be ~8.5.
4. Store at room temperature.

1x TBE working solution

Dilute the stock solution by 10x with deionized water
(dH_2O) in a new 1 L bottle.

Notes:

SOB Medium

It might not be necessary for every group to make up SOB media as only 50 mL is needed for Protocol 5. Make sure that the chemicals below, including 5 M NaOH, and the autoclave are available before starting. Follow the autoclave instructions from the lab teachers. The finished media should be autoclaved within a couple of hours to prevent contaminative growth.

Introduction

SOB medium, or Super Optimal Broth, is used for preparing chemically competent cells. This protocol is adapted from Ausubel *et al.* (1999).

Components

- Yeast extract;
- Bacto™ tryptone;
- NaCl;
- KCl;
- ddH$_2$O;
- 5 M NaOH.

Procedure

Add the following to a 1 L bottle:

1. 0.5% (w/v) yeast extract.
2. 2% (w/v) Bacto™ tryptone.
3. NaCl to a final concentration of 10 mM.
4. KCl to a final concentration of 2.5 mM.

5. Add ddH$_2$O up to 600 mL.
6. Adjust the pH with 120 µL 5 M NaOH.
7. Autoclave for 20 min within 2 hr.
8. Store at room temperature.

Notes:

LB Medium

Depending on the length of time spent in lab, be prepared to make LB several times, either due to high usage or contamination. Make sure that the chemicals below, including 5 M NaOH, and the autoclave are available before starting. Follow the autoclave instructions from the lab teachers. The finished media should be autoclaved within a couple of hours to prevent contaminative growth.

Introduction

Lysogeny broth (LB) is one of the rich media for bacterial growth and is the standard choice for *E. coli*. LB was developed by G. Bertani and later optimized by Luria during the 1950s. It subsequently acquired different names, including Luria broth, Luria–Bertani media and Lennox broth, some containing different salt concentrations.

Components

- NaCl;
- Bacto™ tryptone;
- yeast extract;
- ddH$_2$O;
- 5 M NaOH.

Procedure

Add the following to a 1 L bottle:

1. NaCl to a final concentration of 0.17 M.
2. 1% (w/v) Bacto™ tryptone.
3. 0.5% (w/v) yeast extract.

4. ddH$_2$O to 600 mL.
5. 100 μL of 5 M NaOH (adjusts the pH to ~7.0).
6. Autoclave for 20 min within 2 hr.
7. Store at room temperature.

Notes:

LB Agar Plates and Addition of Antibiotics

Make sure that the chemicals below, including 5 M NaOH, and the autoclave are available before starting. Follow the autoclaving instructions from the lab teachers.

Introduction

These plates contain solidified lysogeny broth (LB), a rich growth medium for *E. coli*. In contrast to growth in liquid LB where the bacteria are mobile, growth on plates gives colonies originating from one single bacterial cell.

Just before pouring the solution into petri dishes, an antibiotic can be added for resistance selection. Normal working concentrations are:

- ampicillin: 100 μg/mL;
- chloramphenicol: 25 μg/mL;
- kanamycin: 50 μg/mL.

Normal stock concentrations:

- 1000-fold higher than above, respectively.

Note: Chloramphenicol stock is dissolved in ethanol.

Components

- NaCl;
- Bacto™ tryptone;
- yeast extract;
- agar;
- ddH$_2$O;
- 5 M NaOH;
- 1000x antibiotic of choice.

Procedure

Add the following to a 1 L bottle:

1. 600 mL LB prepared fresh as above (non-autoclaved).
2. 9 g agar.
3. Shake. It is unnecessary to dissolve all solids now because autoclaving will do this.
4. Autoclave for 20 min within 2 hr.
5. Let it cool to ~40–50°C (touchable, so the antibiotics will not be destroyed by the high temperature).
6. Add 600 µL of 1000x antibiotic of choice (if any) and gently swirl the bottle to mix. Do not shake the bottle vigorously as this will create many bubbles that will be transferred to your plates!
7. Pour into empty petri dishes just enough to cover the surface (~20 mL per plate). If bubbles remain in the plates, heat the plate surface carefully with a burner to burst them. But make sure not to heat the solution in the plate too much since it might degrade the antibiotic.
8. Leave the plates at room temperature to solidify (~1 hr).
9. Solidified plates should be turned upside down for a few hours at room temperature, then stored at 4°C.

Notes:

PROTOCOL 2. OVERNIGHT CULTURES WITH ANTIBIOTICS, AND GLYCEROL STOCKS

Overnight Cultures with Antibiotics

Setting up an overnight culture of a single bacterial strain needs a plate with single colonies and LB containing the appropriate antibiotic.

Introduction

Antibiotics should not be added to LB before autoclaving the LB or after subsequent cooling to room temperature for storage because antibiotics are not sufficiently stable. Antibiotic solutions are stored pure at −15°C and only added just before the addition of bacteria.

Components

Normal working concentrations are:

- ampicillin: 100 µg/mL;
- chloramphenicol: 25 µg/mL;
- kanamycin: 50 µg/mL.

Normal stock concentrations:

- 1000-fold higher than above, respectively.

Note: Chloramphenicol stock is dissolved in ethanol.

Procedure

1. Quickly burn the neck of a bottle containing LB medium before pouring it out into a flask (or tube). Even the

slightest contamination of LB will be visible the next day as an unwelcome culture!

2. Add the antibiotic to give the appropriate concentration listed above.

3. Scoop one colony from the plate with a sterile inoculation loop (or micropipette tip).

4. Immediately stick the loop (or tip) into the flask (or tube) containing the medium and antibiotic for a few seconds.

5. Cover the flask loosely with aluminium foil (or cap tube loosely) by taping it on to allow exchange of oxygen. Good oxygenation is required not only for maximal growth, but also for chemical maturation of the chromoprotein chromophores.

6. Incubate at 37°C with shaking overnight.

Cell Glycerol Stocks

Introduction

Cell glycerol stocks are stored at −80°C with the glycerol helping to maintain the viability of the cells.

Procedure

1. Mix 600 μL of an overnight culture with 400 μL of 50% glycerol (to give 20% glycerol final).

2. Place in the −80°C freezer.

   Note: In contrast to the procedure for storing certain enzymes, a cell glycerol stock should not be cooled by snap freezing in liquid nitrogen.

Plasmid Preparations

Optionally, plasmids can be harvested from overnight cultures with a plasmid miniprep kit according to the manufacturer's instructions.

Notes:

PROTOCOL 3. BIOBRICK™ 3A ASSEMBLY AND GEL ANALYSIS

Introduction

The starting point of every 3A assembly is plasmid DNA preps of three different BioBricks™ (top of Fig. 25). The endpoint is, hopefully, colored bacterial colonies.

Components

- DNA plasmids;
- ddH$_2$O;
- 10x reaction buffer for restriction enzymes provided by manufacturer;
- EcoRI restriction endonuclease, heat-inactivatable;
- XbaI restriction endonuclease, heat-inactivatable;
- SpeI restriction endonuclease, heat-inactivatable;
- PstI restriction endonuclease, heat-inactivatable;
- T4 DNA ligase;
- 10x reaction buffer for T4 DNA ligase provided by manufacturer;
- papers of every different color, as well as black, grey and white.

Procedure

Digestion

1. Make three mixes: each contains 500 ng of one of the three plasmids and ddH$_2$O to 43 µL.
2. To each mix, add 5 µL of 10x reaction buffer for restriction enzymes.

3. Add 1 µL each of the appropriate endonucleases (two per tube) according to Fig. 25 to give a final volume of 50 µL.
4. Tap on the tubes to mix. If necessary, centrifuge for a few seconds to spin down the liquid.
5. Incubate at 37°C for 30 min.
6. Heat-inactivate the enzymes by incubating at 80°C for 20 min.

At this point, samples may be stored at −20°C.

Gel analysis of digests (recommended for first time)

Run 20 µl of each digestion mixture (200 ng) on a 1% agarose gel (Protocol 4) to measure the extent of digestion. Also run the three uncut plasmids (negative controls) directly beside their cut versions, and a DNA ladder marker should be loaded in a middle lane.

After the run, there should be one or more DNA bands visible under UV light in each lane. The marker will help indicate the size of the linear (cut) fragments. The lanes with cut plasmid should contain two bands: the slower one is the vector and the faster one is the insert. If the insert is very small (*e.g.* some promoter parts), it may be invisible due to a low amount of stainable DNA and low resolution or due to running off the gel. The uncut plasmid should be one or two bands: a supercoiled version and a nicked form (one or both of the circular strands of DNA has a break in the phosphodiester chain, thereby allowing relaxation of the coil). Note that the different topologies of linears, nicked circles and supercoils cause each to migrate at a different rate.

Ligation

1. Add 2 µL (20 ng) of each of the three digestion mixtures to 11 µL of water.
2. Add 2 µL 10x reaction buffer for T4 DNA ligase.
3. Add 1 µL of T4 DNA ligase to give a final volume of 20 µL.
4. Incubate at room temperature (~22°C) for 30 min.
5. Heat-inactivate the enzymes by heating at 80°C for 20 min.

At this point, samples may be stored at −20°C.

Transformation

1. This first requires preparation of competent cells (Protocol 5).
2. Competent cells are transformed as described (Protocol 6).
3. The next day, bacterial colonies are evaluated by eye for color. Try placing papers of every different color, as well as black, grey and white, underneath the agar plates to test which background color gives optimal sensitivity for detecting your faintly colored colonies.

Notes:

PROTOCOL 4. AGAROSE GEL ELECTROPHORESIS

Be familiar with the safety procedures in Chapter 4 before starting. Preferably set up the gel equipment at multiple locations to avoid congestion.

Introduction

Agarose gel electrophoresis is used for separation and analysis of larger (>100 bases in length) nucleic acids under non-denaturing conditions. By adding the sample with loading buffer to the gel wells and applying a current over the anode and cathode, the negatively charged nucleic acid will migrate to the positive electrode. Analysis requires that the gel contains a DNA stain visible under UV light (see Chapter 4). Since the stain interacts with nucleic acids and is therefore potentially mutagenic, always wear nitrile gloves when working with agarose gels. If the stain is ethidium bromide, not Sybr®Safe, make sure that contaminated waste (pipette tips, gels, *etc*.) is disposed of in hazardous waste boxes. Use protective glasses when using the UV light box.

Components

- Agarose;
- 1x TBE;
- Sybr®Safe;
- Loading dye mix
- DNA ladder size marker
- DNA samples

Procedure

Casting a 150 ml gel

1. Close the ends of the gel tray either with tape or by a casting stand, depending on the equipment. The gel tray must be on a level surface.

2. Insert the comb into the gel tray at one end ~1 cm from the edge.

3. For a 1% 150 mL agarose gel, weigh 1.5 g of agarose in a 500 mL conical flask. Add 150 mL 1x TBE buffer. If the gel tray is smaller, calculate the amount needed.

4. To dissolve the agarose in the buffer, swirl to mix and microwave for a few minutes taking care not to boil the solution out of the flask. Remove the flask occasionally and check whether the agarose has dissolved completely. Be careful — the solution is very hot! Insulated gloves are too bulky to easily pull the flask out of the microwave, so a folded up paper towel is suggested.

5. Let the agarose solution cool down. Once the solution is touchable, add the DNA stain. Check the stock concentration and add the appropriate amount to give the desired final concentration. The working concentration for ethidium bromide is 0.5 μg/mL while for Sybr®Safe it is simply 1x.

6. Pour the gel solution into the gel tray. Remove any air bubbles with a pipette tip. Put in comb.

7. The gel will solidify while cooling down to room temperature. Depending on the initial temperature, this will take ~30 min.

Running the gel

1. Release the gel tray from the tape or casting stand. Place the gel tray into the buffer chamber and remove the comb carefully.

2. Add 1x TBE buffer until the gel is completely covered.
3. Take part of your DNA samples (~0.2 μg) and mix with loading dye. This can be done either in 1.5 mL tubes or, if the volumes are very small, on a piece of parafilm.
4. Load the size marker mixed in 1x loading dye (~6 μL final volume) into a middle well.
5. Load your samples into the other wells while writing down which lanes have which samples.
6. Put the lid onto the buffer chamber and connect it to the power supply. Make sure to put it in the right direction so that your DNA runs towards the positive (red) electrode.
7. Run the gel at 100 V for 30–60 min. Neither of the two dyes should be run off the gel. If the electrophoresis runs correctly you will notice air bubbles coming from the negative (black) electrode.
8. Stop the run and bring your gel to a UV table to visualize your gel bands. Use protective glasses. If sufficient separation was not achieved, put the gel back into the buffer chamber and run it for longer.
9. Take a picture of your gel.

Notes:

PROTOCOL 5. PREPARATION OF COMPONENT *E. coli* CELLS USING CaCl$_2$

A plate with single colonies of a wild-type strain of *E. coli* will be provided at the start. In case this procedure fails, teachers should have a backup stock of competent cells available until students successfully make their own competent cells.

Introduction

E. coli is inefficient at taking up foreign plasmids. One cannot rely on its natural competence (ability to take up foreign DNA). For increasing the competence, the cell wall is made permeable by treatment with CaCl$_2$. It is important that the whole process is kept chilled! Remember to be careful with the cells as they become very fragile; *e.g.* resuspend cells in microfuge tubes by gentle micropipetting or tapping, not vortexing! *E. coil* DH5α is strongly recommended because of high competence and low ability to degrade foreign DNA by restriction at unmethylated sites.

Components

- SOB medium;
- plate with single cell colonies;
- 0.1 M CaCl$_2$, ice-cold;
- 0.1 M CaCl$_2$ with 20% glycerol, ice-cold;
- liquid nitrogen.

Procedure

1. Take one colony and start a 5 mL overnight culture at 37°C, with shaking.
2. Dilute the overnight culture 1:100 into 50 mL SOB medium.

3. Grow culture at 37°C with shaking to an $OD_{600} = 0.4$.

4. Let the culture sit on ice for ~15 min, swirling occasionally. When the cells are properly chilled, proceed to the next step.

5. Pour the culture into a 50 mL Falcon™ tube.

6. Centrifuge at 3500 rpm for 5 min at 4°C.

7. Remove as much as possible of the supernatant without disturbing the pellet.

8. Resuspend the pellet in 100 μL ice-cold 0.1 M $CaCl_2$ with the help of a sterilized loop.

9. Add 15 mL ice-cold 0.1 M $CaCl_2$. Mix gently by pipetting up and down a few times. Do not vortex!

10. Incubate the cells on ice for 30 min.

11. Pellet the cells again at 3500 rpm for 5 min at 4°C.

12. Resuspend the cells in 2 mL ice-cold 0.1 M $CaCl_2$/20% glycerol.

13. Incubate for 45 min on ice.

14. Aliquot carefully in 50 μL amounts to chilled 1.5 mL tubes.

    Note: Some gentle mixing is required as cells tend to fall to the bottom of the liquid. Also, competent cells give the highest transformation efficiencies when fresh compared with after freezing in Step 15.

15. Snap freeze in liquid nitrogen any tubes that will not be used for transformation within a few hours.

16. Store at −80°C.

    Note: Once the cells have been frozen and thawed, they cannot be refrozen again because this kills the cells.

Notes:

PROTOCOL 6. TRANSFORMATION OF CaCl$_2$-COMPETENT *E. coli* CELLS

This requires competent *E. coli* cells from Protocol 5 or supplied by the teacher.

Introduction

Remember that competent cells are very fragile and should only be mixed gently. Transformation plating results, like most results, can be very difficult to interpret without positive and negative controls. A good way to plan controls is to think how you might interpret different kinds of possible results. For example, are colonies really due to correct clones, or are they due to contaminating cells (due to degradation of the antibiotic plates stored for too long), contaminating plasmids or uncut vector? Is a lack of colonies due to failed ligations or just poor competent cells? **Three plating controls** are generally used to distinguish the above possibilities:

1. Water (a negative control).
2. Unligated, cut plasmid(s) (a negative control).
3. Intact plasmid (a positive control). This transforms much more efficiently than ligated plasmid, so 0.1 and 10 ng is sufficient. This enables calculation of the transformation efficiency of the competent cells. An additional advantage of this control is to compare color intensities of the colonies. Thus, when cloning in the destination vector, use the destination vector as the control. When mutating a chromoprotein gene by PCR, use the plasmid containing the chromoprotein gene as the control.

Components

- CaCl$_2$-competent *E. coli* cells;
- SOB media;
- DNA plasmid.
- Agar plates

Procedure

1. Turn on a water bath or heating block to 42°C.
2. Thaw competent cells on ice for 15 min.
3. Add 5 µL of ligation reaction mixture or controls above to 50 µL of competent cells.
4. Incubate for 30 min on ice.
5. Heat shock for 45 s at 42°C.
6. Incubate for 5 min on ice.
7. Add 950 µL of SOB media (pre-heated to 37°C).
8. Incubate for 1–1.5 hr at 37°C, with occasional gentle mixing by inversion of the tubes.
9. For positive controls, mix gently and plate 100 µL only (=1/10th) on an agar plate containing the appropriate antibiotic as in Step 12.
10. Spin cells down from remaining 900 µL at 4000 rpm for 5 min.
11. Discard all but 100 µL of the supernatant and resuspend the pellet in the remaining 100 µL.
12. Spread the remaining suspension on an agar plate containing the appropriate antibiotic as follows (see fire and burner safety procedures in Chapter 4 before you start):

    (i) Dip the spreader into 95% ethanol.
    (ii) Put it into the flame for a second.
    (iii) Let the ethanol burn off outside the flame.

(iv) Spread the bacterial suspension evenly out on an agar plate. Continue until all the inoculum has gone into the agar.

(v) Put the plates at 37°C overnight.

Following days

13. Day 2: Calculate transformation efficiency (colonies/µg) of the competent cells using the positive control plates. How does it compare with the expected efficiency? In the evening, re-streak appropriate colonies (Protocol 7).

14. Day 3: Set overnight cultures.

15. Day 4: Make glycerol cell stocks (Protocol 2) of strains worth saving and process the rest of the culture according to instructions.

Notes:

PROTOCOL 7. BACTERIAL RE-STREAKING TECHNIQUES

It is worth practicing this method as early as possible in the lab because, although it only takes a few minutes, it takes almost a day to obtain the results and it can be tricky at first.

Introduction

Growth on plates gives colonies originating from one single bacteria cell. However, a single colony is not guaranteed to be clonal, and this is particularly problematic when there are several hundred colonies on a plate. Also, some colonies might not actually be resistant to the antibiotic in the plate (e.g. if they sit next to a colony secreting the ampicillin resistance enzyme). Re-streaking is recommended to address these issues, although it has the disadvantage of taking an additional day. There are many different re-streaking techniques, four of which are given here. Find out which procedure works best for you, or come up with your own method!

Procedure Alternative 1 (Figs. 27 and 28)

1. Take one colony from a plate using an inoculation loop and smear it out on one edge of a new plate.
2. Draw one line vertically through the first lines to the centre of the plate.
3. Sterilize the loop and then go back and forth through the vertical line all the way to the other edge of the plate.

Procedure Alternative 2

1. Take one colony from a plate and streak it out within one quadrant at one edge of a new plate.
2. Sterilize the loop and then draw one line through the first set of lines.
3. Streak new lines at an adjacent quadrant of the plate.
4. Repeat the last procedure twice more to fill all four quadrants.

Procedure Alternative 3

This is similar to Procedure 2 but brings fewer cells to the next streak, more quickly reaching single cells on the plate.

1. Take one colony from a plate and streak it out within one quadrant at one edge of a new plate.
2. Sterilize the loop and start drawing the next line from the end of the previous streak.
3. Repeat the last procedure twice more to fill all four quadrants.

Procedure Alternative 4

This method eliminates so many cells that you can re-streak up to four different colonies on the same plate.

1. Divide a new plate into four labeled quadrants by marking the back of the plate.
2. Take one colony from a plate and swirl the loop around carefully in ~20 µL ddH$_2$O in a 1.5 mL tube.
3. Draw to and fro in one quadrant on the plate.

Notes:

PROTOCOL 8. LYSIS OF *E. coli* CELLS WITH LYSOZYME

The teacher may prepare the Tris-HCl and EDTA stocks beforehand because they require a pH meter.

Introduction

Lysozyme is an enzyme that digests the cell wall, with the main commercial source being hen egg white. Triton®X-100 is a detergent that solubilizes the cell membrane lipids. This protocol is adapted from Sambrook and Russel (2006).

Components

- 1 M Tris-HCl, pH 8.0;
- 0.5 M EDTA, pH 8.0;
- NaCl;
- Triton®X-100;
- lysozyme stock solution at 10 mg/mL in 10 mM Tris-HCl, pH 8.0;
- plate containing single colonies.

Procedure

1. Start overnight cultures of appropriate test and control strains of *E. coli*.
2. Prepare the lysis buffer from the first four components in the list above to give these final concentrations:

   10 mM Tris-HCl, pH 8.0;
   1 mM EDTA;
   100 mM NaCl;
   0.5 % (v/v) Triton®X-100.

3. Pellet 2 ml of an overnight culture in a 1.5 ml tube by centrifugation twice at 5000x g for 5 min. Remove the supernatants.

4. Resuspend the cell pellet in 300 μL of the lysis buffer.

5. Add 25 μL of lysozyme stock solution.

6. Mix by vortexing for a couple of seconds.

7. Incubate the sample at 37°C for 30 min.

   Note: After this incubation, the sample may also be freeze-thawed a couple of times to test if this improves lysis.

8. Centrifuge the sample at max speed for 3 min to pellet the cell debris. Check the color of the pellet as it may tell the effectiveness of the cell lysis.

9. Take the supernatant and quantify its color using a spectrophotometer.

Notes:

PROTOCOL 9. POLYMERASE CHAIN REACTION (PCR)

This protocol introduces PCR and explains how to order and dissolve primers. Protocols 10 and 11 below give details of specific PCR reaction mixes and programs in our workflow.

Introduction

PCR is an *in vitro* method for amplifying and/or mutating DNA (Fig. 31).

Components

- DNA template;
- 2 oligodeoxyribonucleotide primers;
- 4 dNTPs (dATP, dCTP, dGTP, dTTP);
- optimized buffer;
- thermostable DNA polymerase.

Choice of Thermostable DNA Polymerases

Fast polymerases, like the standard Taq polymerase, have a high error rate and do not extend well over long distances. In addition, Taq polymerase usually adds an untemplated A onto the 3′-end of the PCR product. For amplifying long fragments, select a polymerase such as Phusion®HF with high processivity and also proofreading ability to increase fidelity.

Primer Design

The first step is to design and order primers that will work optimally. Primers may be completely complementary to their target template or have 5′ overhangs (*e.g.* to introduce a restriction site, promoter or mutation (see Protocol 10)). Poorly designed primers are the main cause of PCR reactions with incorrectly sized products or no products at all. Primers that hybridize at incorrect positions in your plasmid or in contaminating *E. coli* chromosomal DNA can be avoided by doing a **BLAST program** search to identify all positions in the target DNA that are complementary to a particular primer sequence, but this search is usually unnecessary. Other parameters to take into account are ensuring low probabilities of self-annealing (both intra- and inter-molecular) and annealing to the other primer (primer dimer-formation), which is checked using programs such as the **CLC Main Workbench program.** Although this program is expensive, a free trial version lasting one month can be downloaded by each student: http://www.clcbio.com/products/clc-main-workbench/. The hybridizing region should be at least 18 nucleotides, contain 40–60% G and C overall and the 3′-ends should not have too high a GC-content. The calculated initial melting temperature (T_m) should be between 53°C and 62°C and both primers should have similar melting temperatures. **This calculation should include information about the polymerase**: different polymerases have different buffers which have different salt concentrations which in turn affect T_m. In addition to the CLC Main Workbench program, there are several free programs available for calculating T_m.

Dissolving Oligos

Ordered primers lack terminal phosphate groups and will be received dry in microfuge tubes. Paste the primer information into your lab notebook.

1. First spin down the tube in a tabletop microcentrifuge at maximum speed for 3 min.
2. Look for the DNA pellet at the bottom of the tube. If it is invisible, assume that it is located there.
3. Add sufficient ddH_2O to the bottom of the tube to give a 100 µM stock.
4. Rinse the inside of the tube walls with the water and a pipette tip to ensure that all the DNA is dissolved. Dissolving is not instantaneous, so let the tube stand at room temperature for a few minutes until placing on ice.
5. Store primer stocks at −20°C.

   These stocks are ready to dilute for some PCR applications, but other applications such as inverse PCR mutagenesis (Protocol 10) require enzymatic phosphorylation of the 5' ends.

PCR Reaction

First find a PCR machine with the program desired or enter your own program based on the polymerase manufacturer's protocol, Protocols 10 or 11, and your primers. A typical PCR program is as follows:

1. Melting step, to separate the DNA template strands from each other.

2. Main cycle including:
   - melting step;
   - annealing step;
   - extension step.
3. Repetitions of main cycle 20–30x.
4. Storage step.

Standard controls for PCR are:

1. Reaction without template (a negative control for contamination).
2. Reaction without polymerase (a negative control for primer dimer formation).

Notes:

PROTOCOL 10. INVERSE PCR MUTAGENESIS

Introduction

Inverse PCR mutagenesis (Hemsley *et al.*, 1989) is a flexible and rapid method for plasmid mutagenesis that combines PCR and cloning (Fig. 32). It enables the rapid alteration or substitution of a sequence up to 80 bases long, insertion of up to 80 bases, or deletion of almost any length desired. This protocol uses purified plasmid DNA target, but it is also possible to perform inverse PCR directly on a bacterial colony (see Protocol 11).

Primer Design

Follow the method used for PCR (Protocol 9) as closely as possible, mindful that compromises may be necessary due to the sequence of the target site. Primer length, including the 5′ overhang, should not be longer than ~60 bases to minimize mutations caused by the low fidelity of commercial chemical DNA synthesis. The key design features are that the two primers will extend away from each other and the two 5′ overhangs will become joined together at the mutation site in the gene (Fig. 33A).

Phosphorylation of Primers

Note: The protocol says ATP (ribonucleotide) and not dATP (deoxyribonucleotide).

Introduction

Commercially synthesized primers lack terminal phosphate groups. This is fine for some PCR applications, but

for inverse PCR mutagenesis, a 5′ phosphate group is necessary for incorporation into a phosphodiester bond by T4 DNA ligase to circularize the PCR product into a plasmid.

Components

- ddH$_2$O;
- primer stock, 100 µM;
- 10x reaction buffer A for PNK provided by manufacturer;
- ATP, 10 mM;
- T4 polynucleotide kinase (PNK).

Procedure

1. Prepare the following reaction mixture:

ddH$_2$O	14 µL
100 µM (100 pmol) primer	1 µL
10x reaction buffer A for T4 polynucleotide kinase	2 µL
10 mM ATP	2 µL
10 U/µL T4 polynucleotide kinase	1 µL
Total (5 µM primer ready to use)	20 µL

2. Mix thoroughly, spin down and incubate at 37°C for 30 min.
3. Heat-inactivate the enzyme at 80°C for 20 min.

Inverse PCR with Phusion®HF DNA Polymerase

Introduction

Phusion®HF DNA polymerase has a little endonuclease activity, so the incubation time with the DNA should not be extended. The program is a version of touchdown PCR that increases specificity but still provides a good yield. Two different PCR programs are recommended because long and short primers tend to have annealing temperatures above and below the extension temperature, respectively, in later cycles. The second program ensures that short primers can anneal for the extension reaction.

Components

- ddH$_2$O;
- dNTPs, 2 mM;
- phosphorylated forward primer, 5 µM;
- phosphorylated reverse primer, 5 µM;
- 5x Phusion®HF buffer provided by manufacturer;
- Phusion®HF DNA polymerase;
- DNA template.

Procedure

1. Choose the appropriate one of the PCR programs (i) and (ii) below:

   (i) PCR program if you have **long overhangs**: the differences between this and the following program are highlighted in bold.

Description of Step	Temp. (°C)	Time (hh:mm:ss)	Number of Cycles
Initial denaturation	98°	00:05:00	1x
Denaturation	98°	00:00:30	
Annealing temp. + 4°C	°	00:00:30	2x
Extension	72°		
Denaturation	98°	00:00:30	
Annealing temp. + 2°C	°	00:00:30	2x
Extension	72°		
Denaturation	**98°**	**00:00:30**	
Annealing temp.	**°**	**00:00:30**	**6x**
Extension	**72°**		
Denaturation	**98°**	**00:00:30**	**25x**
Extension	**72°**		
Final extension	72°	00:07:00	1x
Storage	4°	∞	1x

(ii) PCR program if you have **short overhangs** where the combined T_m of the initial priming sequence and overhang does not exceed 72°C.

Description of Step	Temp. (°C)	Time (hh:mm:ss)	Number of Cycles
Initial denaturation	98°	00:05:00	1x
Denaturation	98°	00:00:30	
Annealing temp. + 4°C	°	00:00:30	2x
Extension	72°		
Denaturation	98°	00:00:30	
Annealing temp. + 2°C	°	00:00:30	2x
Extension	72°		
Denaturation	**98°**	**00:00:30**	
Annealing temp.	**°**	**00:00:30**	**30x**
Extension	**72°**		
Final extension	72°	00:07:00	1x
Storage	4°	∞	1x

2. Calculate the extension time in your lab note book:
   - Length of amplicon: _____kb.
   - Phusion® pol extension time per kb: 15–30 s/kb.
   - Extension time (length × extension time per kb):
     _____ hh:mm:ss.
3. Fill in the extension time in your chosen program grid above.
4. Use the manufacturer's T_m calculator and Phusion® information sheet to fill in the annealing temperatures in your chosen program grid above.

   Note: The recommended annealing temperature is *above or equal to T_m* for Phusion® pol, depending on primer length, but *below T_m* for Taq pol!
5. Program the PCR machine (unless an identical program already exists in the machine).
6. Dilute a small amount of your plasmid to 1 ng/µL.
7. Mix the seven components below in a PCR reaction tube (0.2 ml tube, which is smaller than a typical microcentrifuge tube).

ddH$_2$O	23.7 µL
2 mM dNTPs	5 µL
Forward primer (5 µM)	5 µL
Reverse primer (5 µM)	5 µL
5x Phusion® HF buffer	10 µL
Plasmid dilution (1 ng/µL)	1 µL
Phusion® HF DNA polymerase	0.3 µL
Total	50 µL

8. Run the PCR reaction.
9. Analyse a 5 µL aliquot by agarose gel electrophoresis.
10. If a full-length band was visible, purify your PCR product using a PCR purification kit (or gel extraction kit) according to the manufacturer's protocol.

PCR purification kit

This purifies DNA products from PCR reaction components to enable downstream applications. Always consult the manufacturer's protocol because the procedure can differ between kits. Typically:

1. A high-salt solution is added to the PCR reaction mix.
2. The PCR solution is loaded onto a silica mini-column.
3. The mini-column is microcentrifuged to force the solution through.
4. A wash solution is loaded.
5. The mini-column is re-microcentrifuged to remove contaminants.
6. A low salt elution buffer is loaded.
7. The mini-column is again re-microcentrifuged to elute the purified DNA.

Digestion with DpnI

Note: Extended digestion times with DpnI may be beneficial.

Introduction

DpnI only digests methylated DNA sites (Fig. 33B), *i.e. in vivo* synthesized DNA like the original vector that was extracted from *E. coli* in a previous step. The PCR product will, on the other hand, be unmethylated and thus remains intact through the reaction.

Components

- ddH$_2$O;
- purified PCR product;
- 10x FastDigest buffer for restriction enzymes provided by manufacturer;
- FastDigest DpnI.

Procedure

1. Calculate the volume of the purified PCR product containing 500 ng and fill in the table below.
2. Calculate the volume of water to make up to 40 µL.
3. Mix the components below.

ddH$_2$O	µL
DNA from inverse PCR (500 ng)	µL
10x FastDigest buffer	4 µL
FastDigest DpnI	1 µL
Total	40 µL

4. Incubate at 37°C for 3 hr to overnight (despite the recommendation of only 15 min by ThermoScientific™).
5. Heat-inactivate the enzyme at 80°C for 20 min.
6. Use a PCR purification kit to purify the PCR product according to the manufacturer's protocol.
7. Measure the concentration of your PCR product.

Ligation

Introduction

The blunt ends on the inverse PCR product are ligated together by T4 DNA ligase to form a circular plasmid for transformation. Save some unligated, Dpnl-treated, purified PCR product for transformation (a negative control).

Components

- ddH$_2$O;
- purified, DpnI-treated PCR product;
- 10x reaction buffer for T4 DNA ligase provided by manufacturer;
- T4 DNA ligase.

Procedure

1. Calculate the volume of the DpnI-treated PCR product containing 50 ng and fill in the table below.
2. Calculate the volume of water to make up to 50 µL.
3. Mix the components below:

ddH$_2$O	µL
Purified PCR product (50 ng)	µL
10x reaction buffer	5 µL
T4 DNA ligase, 5 U/µL	1 µL
Total	50 µL

4. Incubate for 60 min at room temperature (~22°C).
5. Heat-inactivate the enzyme at 80°C for 20 min.
6. Transform competent cells according to Protocol 6.
7. On the following day, re-streak appropriate colonies.

Notes:

PROTOCOL 11. COLONY PCR

Note that in contrast to Protocol 10, it does not matter whether or not the primers have been 5' phosphorylated. Also Taq DNA polymerase is preferred for colony PCR.

Introduction

The primary goal of colony PCR is to prepare DNA samples from several colonies to screen for the desired mutant by sequencing (Fig. 34). DNA samples for sequencing are more easily prepared by colony PCR reactions than by overnight cultures followed by plasmid preps. There is no need for a cell lysis step because the DNA template is released from the bacterial colony during a PCR reaction. Before sending for sequencing, PCR products are sized and quantified on an agarose gel to verify that the PCRs worked. Also, if a large deletion or insertion was desired, this size difference may be visible on the gel as a preliminary screening step before sequencing (provided that you also run a **control PCR reaction** for comparison!).

Components

- A plate with re-streaked, single colonies;
- ddH$_2$O;
- dNTPs, 2 mM;
- VF2 primer;
- VR primer;
- 10x Taq PCR buffer provided by manufacturer;
- Taq DNA polymerase.

Procedure

Screening requires testing several different colonies at once, *i.e.* several PCRs. In such cases, instead of preparing several reaction mixes individually, the number of pipetting steps can be reduced and the reproducibility of the reactions increased as follows: prepare just one "**master mix**" that includes all components except the variables (in this case, the cells). For example, if you decide to perform six PCRs (including your original control colony and two negative controls), then you will need to make up a volume of master mix equivalent to seven reaction mix volumes, not six volumes, because there never seems to be enough volume in the last aliquot! So instead of using the volumes given for the reaction mix in Step 6 below, you would use 7x as much of each volume to make up your master mix, then aliquot 49 µL six times into different 0.2 mL tubes. The remaining volume-deficient "seventh aliquot" is then discarded.

1. Calculate the extension time in your lab note book:
   - Length of amplicon: _____ kb.
   - Taq extension time per kb: 1 min/kb.
   - Extension time (length × extension time per kb): _____ hh:mm:ss.
2. Fill in the extension time in the program grid:

Temperature	Time (hh:mm:ss)	Number of cycles
94°	00:05:00	1x
94°	00:00:30	
58°	00:00:30	30x
72°		
72°	00:07:00	1x
4°	∞	1x

3. Program the PCR machine (unless an identical program already exists in the machine).

4. Number the colonies you wish to test by marking on the backs of test and control plates.

5. Using a sterile loop, pick a small portion of each of these colonies and suspend individually in 30 µL of water. Mix thoroughly.

6. Calculate the volumes for making up your pre-mix for several reactions (as described above) based on the volumes provided for just one reaction here:

ddH$_2$O	28.7 µL
10x Taq PCR buffer	5 µL
2 mM dNTPs	5 µL
VF2 (5 µM)	5 µL
VR (5 µM)	5 µL
Taq DNA polymerase	0.3 µL
Total reaction mix	49 µL

7. Prepare your pre-mix and divide into 49 µL aliquots on ice.

8. Add 1 µL of each cell suspension to each aliquot and mix.

9. Run the PCR reactions.

10. Analyze a 5 µL aliquot of each PCR by agarose gel electrophoresis.

11. For reactions where a full-length band was visible, purify the PCR product using a PCR purification kit according to the manufacturer's protocol.

12. Measure the concentrations of your PCR products.

Notes:

PROTOCOL 12. GIBSON ASSEMBLY

Introduction

This new PCR-based method (Gibson *et al.*, 2009) joins multiple DNA parts in a manner independent of DNA sequence. The 5′ ends of the primers are designed to include 20–150 base overlaps with the fragment that they will be ligated to (Fig. 38). The difficulty increases with the number of parts in the reaction; up to 12 were reported by Gibson *et al.* Each DNA can be up to several hundred kilobase pairs in length and should not be less than ~250 bp because of the risk of complete degradation by the exonuclease. Shorter fragments can be tried with less endonuclease.

Components

5x Isothermal Reaction Mix:

ddH$_2$O	mL
1M Tris-HCl, pH 7.5	3 mL
2 M MgCl$_2$	150 μL
1 M DTT	300 μL
100 mM NAD	300 μL
100 mM dATP	60 μL
100 mM dCTP	60 μL
100 mM dGTP	60 μL
100 mM dTTP	60 μL
PEG-8000	1.5 g
Total	6 mL

Aliquot in 320 μL portions and store at −20°C.

Assembly Master Mix:

ddH$_2$O	mL
5x Isothermal Reaction Mix	320 µL
10 U/µL T5 exonuclease	0.64 µL
2 U/µL Phusion® HF DNA polymerase (normal version, not hot-start version)	20 µL
40 U/µL Taq DNA ligase	160 µL
Total	1.2 mL

Aliquot in 15 µL portions and store at −20°C for a year or more.

Procedure

1. Perform PCRs (Protocol 9 with unphosphorylated primers) with your target DNAs and Phusion® HF DNA polymerase.
2. Gel purify the PCR products with a kit (Table 4).
3. Thaw a 15 µL aliquot of Assembly Master Mix.
4. Add 5 µL of ca. equimolar mixture of three DNAs. Use 10–100 ng of each ~6 kbp DNA fragment.
5. Incubate at 50°C for 15–60 min, where 60 min is optimal.
6. Transform into competent cells (Protocol 6).

Trouble-Shooting by Overlap Extension PCR

Some overlaps seem to be more problematic than others, perhaps due to secondary structure formation at 50°C (*e.g.* overlaps that contain terminator stem loops). If a particular junction is hard to obtain by Gibson assembly, it can first be obtained by **overlap extension PCR** (OE-PCR or SOE-PCR), which is PCR of the two

fragments together without primers (Fig. 39). For OE-PCR, use enough DNA (>200 ng) to see bands on a gel to make sure it worked. Then use the joined fragment in a standard Gibson assembly.

Notes:

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7

Advanced Methods

FLOW CYTOMETRY AND CELL SORTING

Flow cytometry is a powerful technique for analyzing fluorescent microscopic particles such as bacteria and eukaryotic cells (Figs. 40 and 41).

Unlike a fluorometer that measures the average fluorescence of a population of cells, flow cytometry gives the actual distribution of fluorescence among individual cells in a population. The cells are suspended in a narrow stream of fluid such that only one cell passes through the laser beam at a time. Each time that happens, the cell scatters the light slightly, both in parallel with the beam (forward scatter) and perpendicular to it (side scatter). Also, if the cells contain fluorescent molecules, they will be excited into emitting light of a different color than the
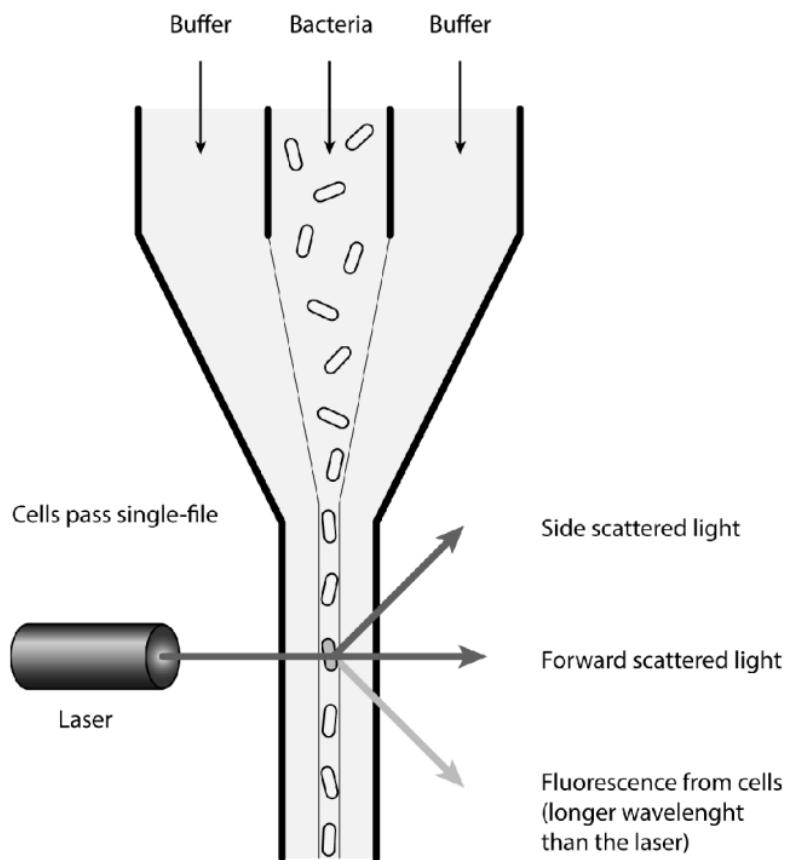
Fig. 40 Schematic of analysis of fluorescent cells by flow cytometry. With respect to chromoproteins, the technique only works with those that can fluoresce to some extent.

laser. A set of detectors in the instrument measures both light scattering and fluorescence, giving detailed quantitative information about individual cells in the population analyzed. Since thousands of cells can be analyzed per second, large amounts of data can be acquired from each sample, giving good statistical accuracy of the measurements. Reporters such as GFP can be used both for labeling of cells and as reporters of gene expression. Fluorescent markers with different colors make it possible
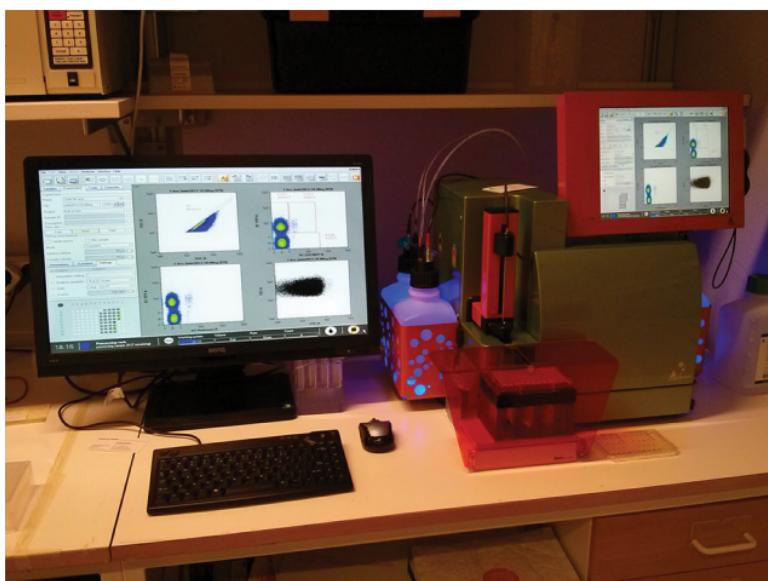
Fig. 41 Flow cytometer machine.

to measure the expression levels of several different genes in a single cell simultaneously, or to track different types of cells in a mixed population. With respect to chromoproteins, the technique only works with those that can fluoresce to some extent: the only two in our chromoprotein parts being amilGFP and amajLime (Table 1 in Appendices). However, we also list two fluorescent proteins (Table 1 in Appendices), Super Yellow Fluorescent Protein 2 (SYFP2; Kremers *et al.*, 2006) and Blue Fluorescent Protein (mTagBFP; Subach *et al.*, 2008), as these are ideal for flow cytometry.

Some flow cytometers can sort the analyzed cells into different containers based on the optical characteristics of each cell, a technique called fluorescence-activated cell sorting (FACS; Fig. 42).

An application of FACS used by Uppsala iGEM students is diagrammed in Fig. 50 below.

Fig. 42 Fluorescence-activated cell sorter (FACS) machine.

RECOMBINATION IN PLASMIDS AND THE CHROMOSOME

Recombination-based *in vivo* methods are essential for adding, subtracting or swapping genes on chromosomes, but they are also competitive with restriction-enzyme-based methods for manipulating plasmid DNAs.

Choosing between gene expression from plasmids versus from the chromosome

There are many benefits of using plasmids when constructing genetic devices in bacterial chassis. Plasmids can be easily prepared from bacteria, they can be cut with restriction enzymes for screening and for assembly of different parts, they are easy to move between different bacterial strains, and plasmids with high copy numbers allow very high expression levels of

genes when needed. Plasmids are also very useful for storage and distribution of genetic parts. However, bacteria can sometimes lose plasmids, especially when they contain highly expressed parts or other constructs that are costly or even toxic for the cells. Plasmids can be more or less stable depending on their origin of replication, but in general it is necessary to use constant antibiotic selection to ensure that the plasmids are not lost. Some plasmids can also have high variation in their copy number, which could be problematic if specific expression levels are critical.

An alternative to expressing genes from plasmids is to integrate them on the bacterial chromosome. This gives single-copy expression levels and higher genetic stability, obviating the need for antibiotic selection. Some integration methods require antibiotic resistance markers during the construction stage, but these can be removed later through use of site-specific DNA recombinases (see below). Removal of antibiotic resistance markers is a requirement for some medical and environmental applications.

Lambda Red Recombineering

The word "recombineering" is derived from "recombination-mediated genetic engineering." Recombineering is a technique that can be used to "knock out" (Fig. 43), replace (Fig. 44), modify or insert genetic material on the bacterial chromosome or on a plasmid.

Unlike cloning and assembly methods that use restriction endonucleases and DNA ligases, recombineering

1. PCR amplify resistance gene using primers with 40 bp overhangs homologous to target gene flanks.
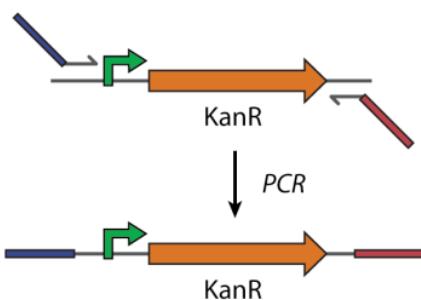
2. Electroporate into strain expressing Lambda Red system.
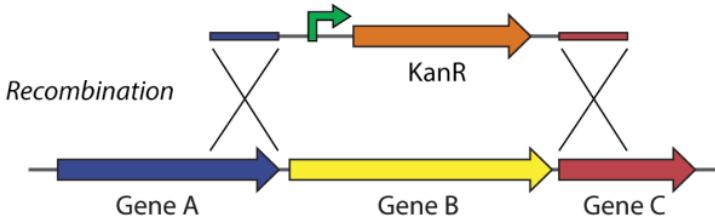
3. Positive selection of antibiotic resistant transformants.
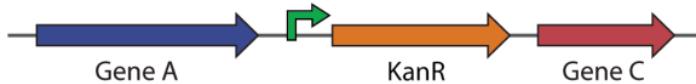
Fig. 43 Knocking out a gene (yellow) by recombination with an antibiotic resistance gene (orange).

does not require conveniently positioned restriction sites; it is instead based on sequence homologies. This makes the technique similar to other homology-based methods such as Gibson assembly, but while Gibson assembly uses enzymes to join different sequences *in vitro*, recombineering is done *in vivo*. In general, the constructs to be integrated are generated by PCR using primers with overhangs containing homologies to the target location.

1. PCR amplify gene of interest together with resistance gene
   using primers with 40 bp homologies to the target.
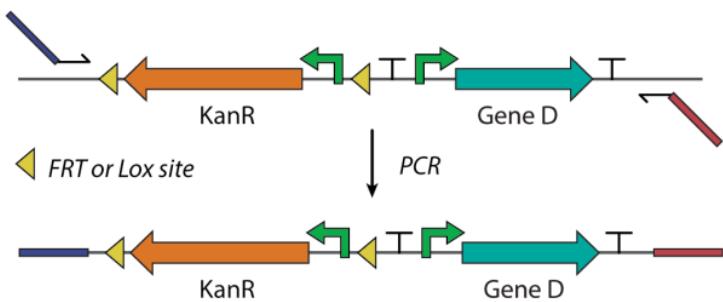
2. Electroporate the targeting construct into strain expressing the
   Lambda Red recombination system.
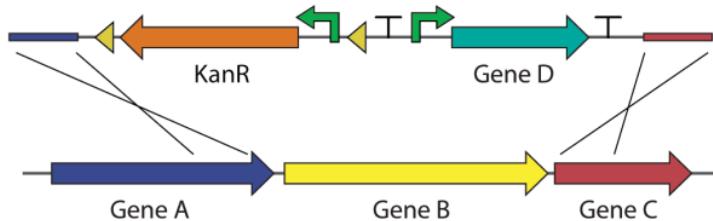
3. Positive selection of antibiotic resistant transformants.
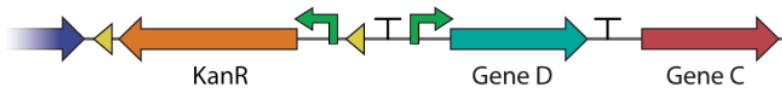
4. Eliminate resistance cassette using site specific recombination
   (FLP or Cre recombinase expression plasmid).
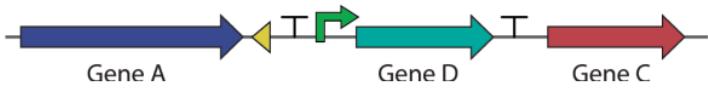
Fig. 44 Replacing a gene (yellow) by recombination with an antibiotic resistance gene (orange) and replacement gene (green). The resistance gene is then removed by site-specific recombination between the short sequences represented by two yellow triangles (see also Fig. 47).

There are some limitations to recombineering systems with perhaps the biggest one being the construct size. While the integration of 2–4 kbp is highly efficient, constructs above this size will have reduced integration frequency. To be able to select successful recombinants, the linear construct must contain a selectable marker, preferably a marker flanked by FRT or lox sites (see below) if the marker should be removed using site-specific DNA recombinases after integration of the construct. Since the integration method is based on homology, it is also critical that the ends of the linear construct are the only sequences homologous to the chromosome or any other genetic material present in the recipient cells. All homologies longer than 20–30 bp besides the targeting sites could give unpredictable integration, and this could be problematic since many commonly used standard parts such as terminators and promoters are derived from native *E. coli* sequences. When constructing systems that are intended to be integrated on the chromosome with recombineering, the use of synthetic parts or parts derived from other organisms could help avoid this problem.

How Lambda Red recombineering works

The Lambda Red recombineering system works in *E. coli* and *Salmonella* and is based on the three "Red" proteins from bacteriophage Lambda that promote homologous recombination:

1. Protein "Gamma" prevents the *E. coli* nucleases RecBCD from degrading the linear DNA.
2. Protein "Beta" binds to single-stranded DNA to promote annealing.

3. Protein "Exo" is a 5′ to 3′ exonuclease that chews back on the ends of the linear DNA to make it single stranded.

The genes for these three proteins can be expressed from a thermosensitive, low copy plasmid such as the pSIM-plasmids (Datta *et al.*, 2006). In these plasmids, the expression of the three Red genes are controlled by a temperature-sensitive repressor that allows tight repression at 30°C. Strains carrying these plasmids are grown at 30°C except when you want to integrate a new construct. Induction of the system at 42°C for 15 min is enough to make the bacteria express a pulse of Lambda Red proteins, making them able to integrate linear DNA. The cells are then made electrocompetent (see below), and the linear construct can be transformed into the bacteria by electroporation. Next, cells are allowed to recover for a few hours before plating on selective media, where only cells that have successfully integrated the synthetic construct will be able to survive. The pSIM plasmid carries a thermosensitive origin that can replicate only below 37°C, so to cure the strain from the plasmid after successful integration, the strain is grown at 42°C overnight. Another common method for chromosomal integration is the Tn7 transposon system.

Single-Stranded Recombineering and Multiplexed Automated Genome Engineering (MAGE)

Although the Lambda Red method above uses double-stranded DNA generated through PCR, recombineering can also be done using synthetic single-stranded oligodeoxyribonucleotides. Unlike double-stranded recom-

bineering, no exonuclease activity is needed to make the mutagenic DNA single stranded, and expression of just protein "Beta" is sufficient for efficient recombination (Fig. 45).

The mutation efficiency depends on both the type of mutation attempted and the oligo design. Since the mutagenic oligo will anneal to the lagging strand of the replication fork, it is important to design it to target the correct strand of the genome. Given that replication is bidirectional from the origin of replication, the strand targeted depends on the position of the locus compared to the origin (Fig. 45). An efficient oligo is generally about 90
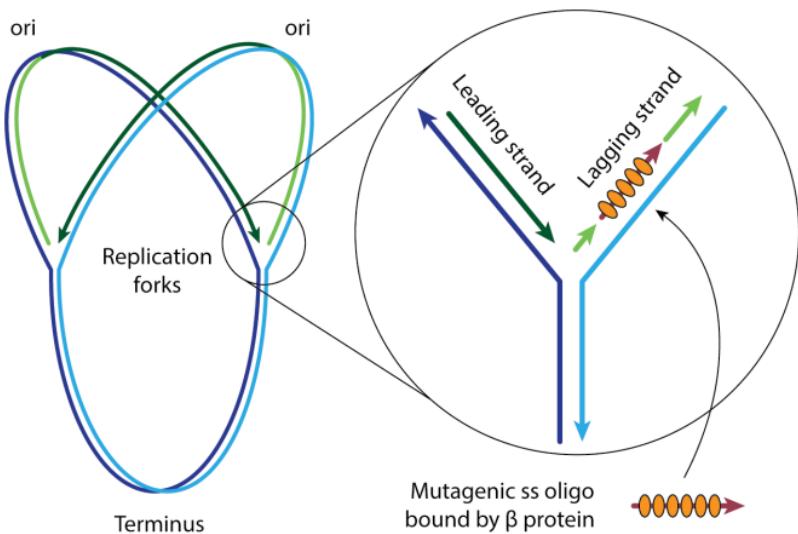

Fig. 45. Single-stranded recombineering. *Left*: chromosomal replication in *E. coli* is bidirectional, starting at the origin of replication (oriC). For single-stranded recombineering, it is important to target the strand templating synthesis of lagging-strand DNA. *Right*: Beta protein (orange) binds to an oligo (red) electroporated into the cell and promotes annealing of the oligo to a lagging-strand target location exposed on the chromosomal replication fork. The mutagenic oligo is then incorporated between Okazaki fragments into the nascent DNA.
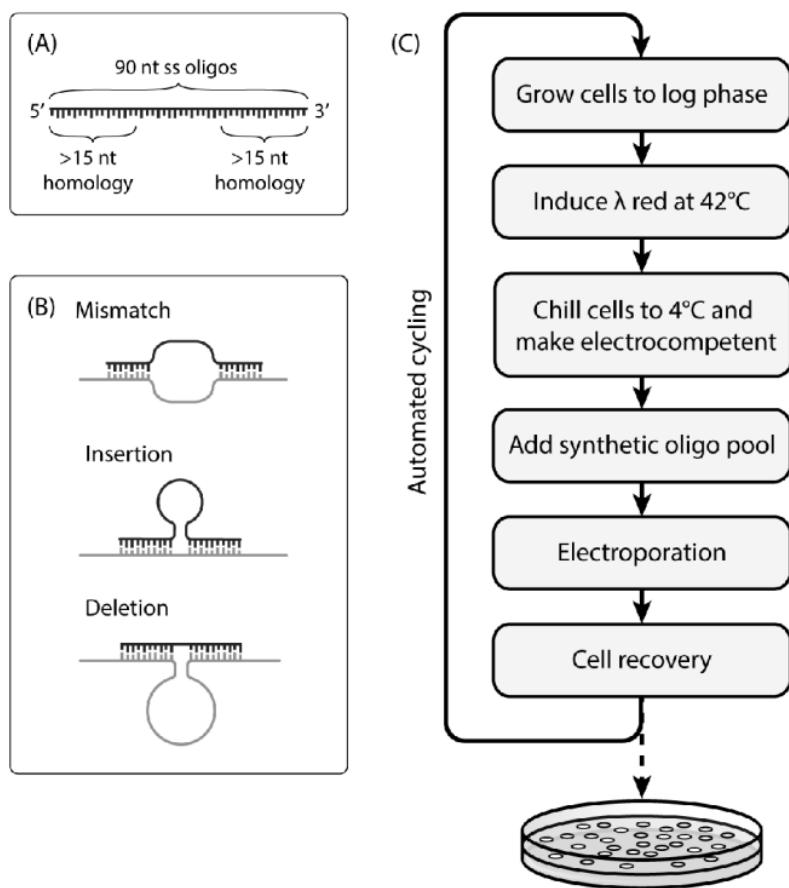
Fig. 46. Synthetic oligo design for single-stranded recombineering and multiplexed automated genome engineering (MAGE). (**A**) Optimal oligo length and homologies with target sequence. (**B**) Oligo designs for three types of mutations. (**C**) MAGE. The fraction of mutant bacteria in the population increases with each cycle.

nucleotides long with at least 15-nucleotide homologies to the target at each end of the oligo (Fig. 46A).

The longer the homology to the target, the more efficient the incorporation will be. Nucleotides in the middle of the oligo introduce mutations by mismatch or insertion or deletion (Fig. 46B). Deletion efficiencies decrease with increasing deletion size. It is also important to consider

secondary structures of the oligo as strong hairpins lower efficiencies. Efficiencies can be further increased by substituting the first four phosphodiester linkages on the 5′ end of the oligo with phosphorothioates to protect from exonuclease degradation.

Single-stranded recombineering is much more efficient than the double-stranded version, so the single-stranded version has the advantage that it is independent of selectable markers and does not leave any "scars" such as FRT sites on the chromosome. However, compared to double-stranded Lambda Red, the size of insertion is limited by the size of the oligo.

The high efficiency of single-stranded recombineering has enabled fast, highly parallel, genomic engineering using a mix of many mutagenic oligos targeting different chromosomal loci and repeating many times (Wang *et al.*, 2009). This method is termed multiplexed automated genome engineering (MAGE; Fig. 46C).

Site-Specific Recombination

Site-specific recombination techniques use a class of enzymes called recombinases that catalyzes the recombination between two short, specific DNA sequences. They are useful for removing a selection marker used for chromosomal integration and for constructing a switch that changes the direction of a promoter under controlled conditions *in vivo*. Two of the most common systems are the Cre/loxP system derived from a bacteriophage, and the Flp/FRT system derived from yeast. The Cre recombinase will recognize sequences called loxP, while the Flp recombinase will bind to sequences called FRT ("flippase recognition target"). Recombinases are fast, specific and efficient, and genetic segments flanked by such sequences
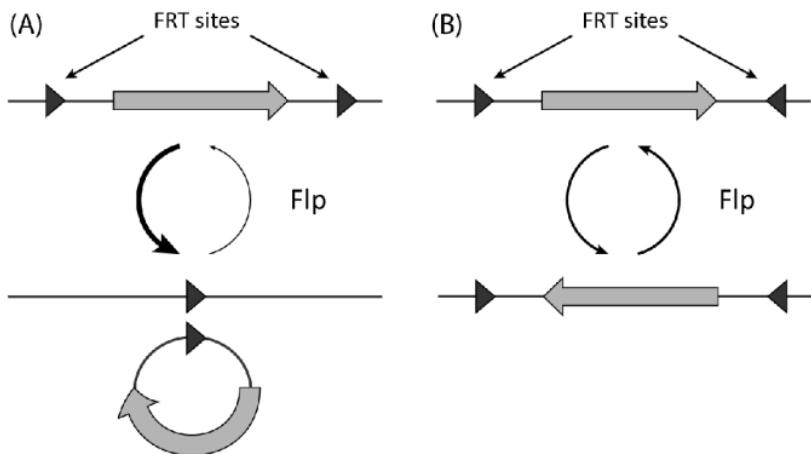
Fig. 47 Site-specific recombination by FRT recombinase. Depending on the orientation of the two short recognition elements (triangles), genetic segments flanked by them are either (**A**) excised (and lost), or (**B**) inverted.

will either be excised (and lost) or inverted, depending on the orientation of the recognition elements (Fig. 47). Scarless methods have also been developed.

Other Recombination Methods

Two other common methods based on recombination should also be mentioned. Bacteriophage transduction is useful for moving large chromosomal fragments between bacteria. And yeast can be transformed with small or large fragments for extra-chromosomal recombination (Oldenburg *et al.*, 1997) as an *in vivo* alternative to Gibson assembly.

ELECTROCOMPETENT CELLS

When using linear DNA fragments to transform *E. coli* during recombineering, higher transformation efficiencies are required than obtainable by the heat shock method, so electroporation is used instead. The protocol for
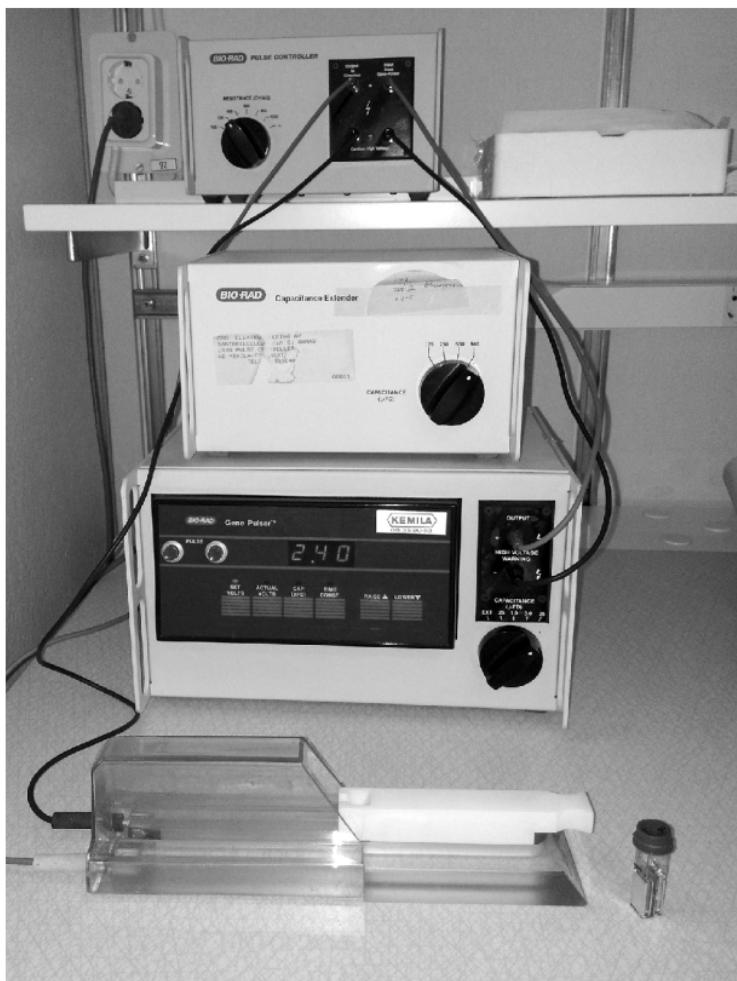
Fig. 48 Electroporation apparatus. The disposable/reusable cuvette on the right is pushed into the left-hand side of the container.

making *E. coli* electrocompetent is very similar to the protocol for making them chemically competent, but the cells are washed with ice-cold distilled water or 10% glycerol instead of calcium chloride. The electrocompetent cells are then mixed with the DNA in an electroporation cuvette, and an electric pulse is passed through the cuvette using a voltage of ~2 kV (Fig. 48).

The cells are then allowed to recover and plated on selective agar, similar to transformation by heat shock.

8

The International Genetically Engineered Machine (iGEM) Competition

The amazing phenomenon known as iGEM was introduced in Chapter 1, and three prize-winning early iGEM projects were summarized in Lab section 4 of Chapter 5. Here we provide more practical information, including chronological blow-by-blow descriptions of the 2011 and 2012 Uppsala iGEM projects that inspired this manual. This illustrates how iGEM teams work from start to finish and also provides the origins and details of the chromo-protein and antisense genes used in this lab course.

iGEM is growing so rapidly that organizational details change significantly from year to year. In 2013,

there were 30 teams competing in a high school division, and for the first time the collegiate division of 204 teams was divided into "undergrad" and "overgrad" competitions. For 2014 and 2015, the collegiate regional and championship jamborees will be combined into one giant jamboree at MIT, with 2500 attendees anticipated. Given that this large number does not include team participants unable to travel to the event, that there are now ~15,000 iGEM alumni, that every team reaches out to the public as part of the competition, and that teams are spread all over the world, the global impact of iGEM should not be underestimated!

HOW TO START AN iGEM TEAM

Starting an iGEM student team is like starting a PCR reaction:

- It starts at the beginning of the cycle (early each year).
- It consists of just a few defined components (team members).
- It needs planning (proposing an action plan and project).
- It involves priming (some help from advisors and an institution).
- It costs a little money (*e.g.* registration fee due ~April 15 for the collegiate division).
- It requires incubation of the components to react (working in a lab).
- It gets underway with copying (standard methods, reagents and equipment).
- It produces something new, amplifiable and useful (useful new organisms).

Detailed practical information on getting started is available from the iGEM webpages, such as http://igem.org/Start_A_Team and http://igem.org/Videos/Lecture_Videos.

Prior iGEM team members should be consulted as early as possible. They will not only show you the ropes, they will likely advise that their team ran out of time because they did not start early enough or they did not expect commercial DNA synthesis to take so many weeks or their experiments did not work as planned.

Faculty should also be approached early. iGEM fund-raising and insurance problems were solved at Uppsala University by faculty offering to turn iGEM into an official university course, an atypical move for the iGEM competition. Although iGEM is, by its very nature, student driven, this does not mean that instructors and advisors should not be exploited, especially ones in your chosen research area. Bounce your project brainstorming ideas off them, run your primer designs by them, and solicit their feedback on drafts of your wikis, posters and presentations early enough to make a real difference.

There is no single recipe for success. Students may pick a project largely because it is related to work performed in a research lab at the same institution or related to the institution's previous iGEM project. Such a project is less difficult to get (re-)started, get supported, get completed in the course of one summer, and even published. Publication is an important quality goal of iGEM, as only publication in a peer-reviewed scientific journal provides strong evidence for completion, novelty and utility together with availability of methods and materials sufficient for others to repeat and build upon it; *e.g.* see http://2012.igem.org/IGEM_Publications.

But if the project is too closely related to existing research, student creativity will be less evident, and in reality such a project may not be easier to bring to fruition. For example, two of the last three Uppsala iGEM teams included among their project proposals ribosomal incorporation of multiple unnatural amino acids into peptides *in vitro*, in part because of our synthetic biology research program in this field already in place. But we advised that our system is not straightforward for inexperienced students to set up and master in a summer, and that *in vitro* replicating systems are generally harder to engineer in a novel way than *in vivo* ones. Indeed, almost all iGEM projects are *in vivo* ones, and the *in vivo* projects proposed and developed by the Uppsala iGEM students, outlined below, turned out very well.

UPPSALA iGEM 2011 — SHOW COLOR WITH COLOR

http://2011.igem.org/Team:Uppsala-Sweden

In spring 2011, the third straight Uppsala University iGEM team formed from 18 undergraduate students, students mostly enrolled in a five-year bachelor/master degree program in Molecular Biotechnology. For the first time, the competition was made into an official course in synthetic biology to guarantee work and travel insurance, partial university funding, and summer accommodation assistance for the students.

After many discussions amongst the students, the team decided to go for a project based on bacterial gene regulation by light. Compared to more traditional ways of inducing gene expression in bacteria, such as adding chemicals like IPTG, light is cheaper and can be regulated more precisely in time and space. To show a proof of

principle, **the team planned to construct a bacterial photographic color film**. Although Uppsala was not working in this area, the basic concept had already been shown elsewhere with the first black and white "coliroid" photo (see "Picture this" in Lab section 4 of Chapter 5). The idea was later expanded to show that multichromatic control of gene expression was possible by using two **light sensors** reacting to two different wavelengths of light, **red** and **green**. The Uppsala team wanted to further expand this to enable complete spectral color readout by adding a third light sensor, just as our eyes and color TVs use three different colors of light. To make the bacteria behave like a photographic film, each light sensor, reacting to different colors of light, would be connected to a different reporter that would make the bacteria change color when illuminated.

The genes for two light sensors were obtained from the Voigt lab: the cph8 gene product reacts to red light and ccaS senses green light. Only one sensor remained: how could the blue light be detected? A few different sensors were considered, but the decision was made to use a **blue** sensor called YF1, since it seemed to have better dynamic performance than the others. Unfortunately, the team was unable to obtain the physical DNA of the YF1 gene and its response regulator, fixJ, because of issues with a material transfer agreement. Instead, the sequences were reconstructed based on the publication and gene synthesis by GenScript, Inc.

Now the team needed a good **color output** system. After looking into what was available from the Registry of Standard Biological Parts, it became clear that the only available color reporters were the ones constructed by Cambridge's 2009 iGEM team in their "*E. chromi*" project (see Lab section 4 of Chapter 5). While this project did provide a way to color bacteria, the pigments were all

based on small molecules and required several different genes for the metabolic pathways to produce them. This would make a multichromatic system very large and complex. The ideal solution would be a one-gene–one-color system. The solution came in an article published in the same year where coral chromoproteins had been expressed to color two different strains of bacteria. One of these chromoproteins, amilCP, was **purple-blue**. The other, amilGFP, was **bright yellow** and could work as the output of the green sensor. The bacterial strains were obtained from the Miller lab and the chromoprotein genes were amplified by PCR, cloned into standard BioBrick™ plasmids, and finally mutagenized to remove a few illegal restriction sites. Expression of these two new genes gave exactly the kind of color output that the team needed. That meant that two colors were ready to use, blue and yellow, while the third color, **red**, already was available in the iGEM distribution kit in the form of mRFP1.

The final system was designed to be modular, so it would be possible to build and test each input and output module separately. Though assembly of the six modules required some 20 BioBrick™ assemblies, all were completed successfully. However, as soon as the modules were assembled, the team discovered several problems with the system. One was bad dynamics of the green sensor: the output promoter PcpcG2 was leaky and gave rather high output signals even when the green sensor was not activated. Also the red sensor was problematic: it used a native *E. coli* system for regulation, so before it could be tested, the native gene had to be knocked out. Even the promising blue light sensor YF1 had some issues: it showed a rather high fitness cost when

expressed, making the bacteria grow very slowly. Still, using a system that linked the blue light input module to a **blue fluorescent** protein output module, it was possible to show that illumination with blue light did indeed activate the output module.

After presenting the project to the iGEM judges during the European Jamboree in Amsterdam in October, the team was selected as one of the finalists that went to the world championships at MIT.

Late during the project, the team garnered a new sponsor, the Korean gene synthesis company Bioneer, and based on the successful use of the amilCP and amil-GFP chromoproteins, the team decided to synthesize a few more promising chromoprotein genes for future use (Fig. 49).
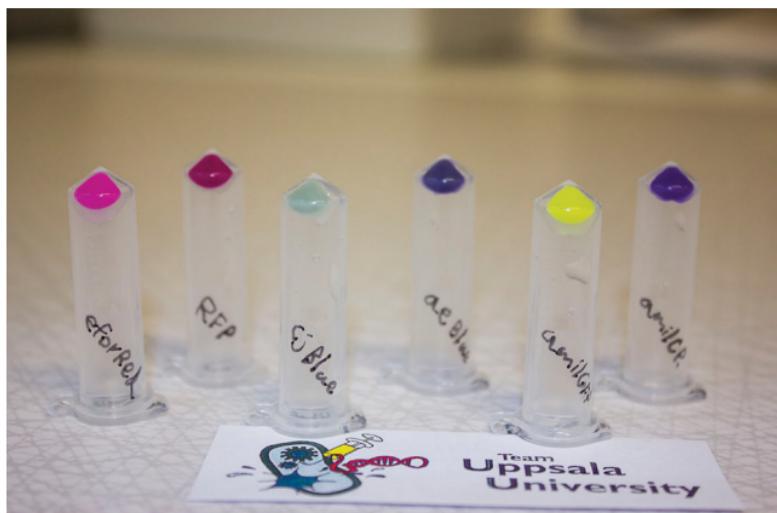

Fig. 49 RFP and some of Uppsala iGEM's designed collection of BioBrick™ chromoproteins. Left to right: pellets of *E. coli* expressing eforRed, RFP, cjBlue, aeBlue, amilGFP and amilCP. (Photograph by A. Gynnå and P. Yuen.)

UPPSALA iGEM 2012 — RESISTANCE IS FUTILE

http://2012.igem.org/Team:Uppsala_University

In spring 2012, another Uppsala University iGEM team formed from 26 undergraduate students. This team went for a completely different project than the year before. The students chose the medical track, focusing on the increasing problem of antibiotic resistance. Would it be possible to construct a system using synthetic small regulatory RNAs (sRNAs; Chapter 2) that could shut down expression of resistance genes, and use this to make antibiotic-resistant bacteria sensitive once again? If such a system could be spread through a bacterial population using a bacteriophage virus, could it be used as an adjuvant to antibiotics?

Building on an approach previously described, the team linked the 5' untranslated region and the first few codons of different clinical resistance genes to a reporter gene, SYFP2 (Kremers *et al.*, 2006; top right of Fig. 50).

This enabled screening for sRNAs that could down-regulate expression of the gene. Large random sRNA libraries were generated by BioBrick™ cloning of the native *E. coli* sRNA, spot42 (BBa_K864440, Table 1 in Appendices), and then randomizing its mRNA-binding region by mutagenic PCR (top left of Fig. 50; see Fig. 1b right of Sharma *et al.*, 2011, and also Figs. 11A and 36). These random libraries were transformed into bacteria carrying the reporter gene (SYFP2 translationally fused to the resistance gene), and bacteria showing down-regulation of SYFP2 were sorted out using fluorescence-activated cell sorting (FACS). These cells were then plated on selective agar and visually inspected on a
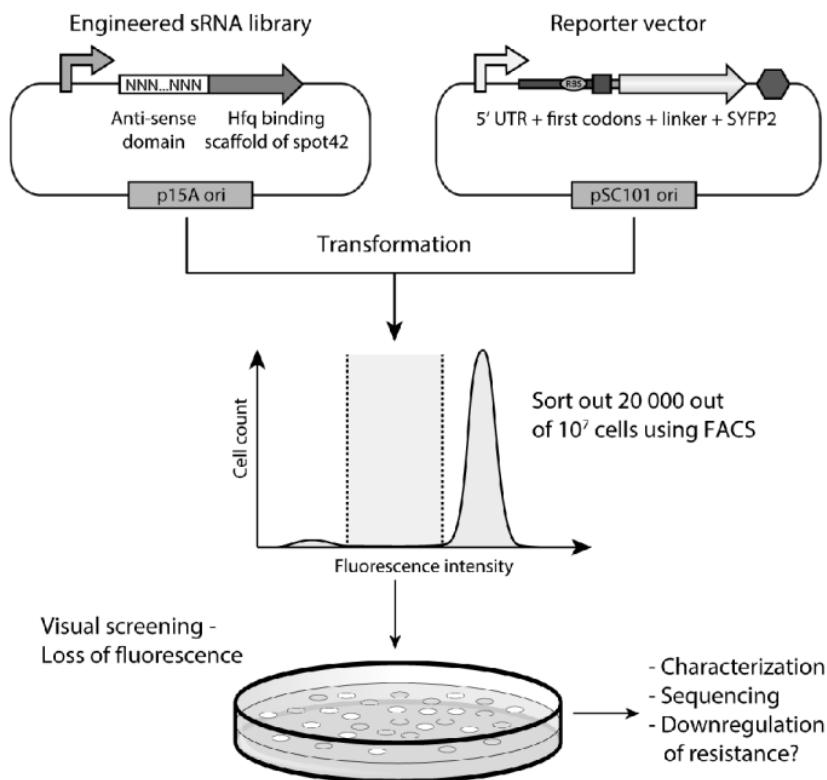
Fig. 50 Screening sRNAs by FACS. *E. coli* was co-transformed with plasmids encoding an sRNA library (top left) and a fluorescent reporter protein fused to the first few codons of the target protein of interest (top right). Only cells with an intermediate fluorescence intensity (<0.5% of cells) were selected by FACS (middle) for plating (bottom).

Visi-Blue transilluminator table. Using 460–470 nm blue light, decreased fluorescence of the down-regulated SYFP expression was seen for many colonies! Plasmids encoding sRNAs were prepared from the isolated clones and re-transformed into the strain carrying the reporter gene to verify that the observed lowered fluorescence was actually due to sRNA down-regulation and not due to loss-of-function mutations in the reporter gene. Those

sRNAs that still showed efficient down-regulation of expression were sent for sequencing, and based on the results it was possible to predict the sRNA-mRNA base-pairing interactions through computer modeling. Successful clones were finally transformed into bacteria carrying actual clinical resistance plasmids, and their antibiotic resistances were measured. The results were promising, with some of the sRNAs lowering the resistance of the bacteria by more than 90%. This approach was later successfully adapted to chromoproteins in the synthetic biology course (Fig. 36).

As a side project, because the chromoproteins from the previous year were requested by several other iGEM teams, additional interesting coral genes were synthesized to expand the Uppsala chromoprotein collection.

UPPSALA iGEM 2013 — LACTONUTRITIOUS — IT'S DELICIOUS

http://2013.igem.org/Team:Uppsala

The 26-student-strong 2013 Uppsala iGEM team picked another completely different project, engineering edible probiotic bacteria to improve their nutritious value. In the European Jamboree in Lyon, the team won best natural BioBrick™ for tyrosine ammonia lyase (BBa_K1033000) and made the finalists to go to MIT. Tyrosine ammonia lyase is an enzyme that synthesizes *p*-coumaric acid from tyrosine, opening up the phenyl-propanoid pathway for *E. coli*.

Again as a side project, the team further expanded the BioBrick™ chromoprotein collection. In this collection at the time of writing, **all Uppsala iGEM chromoproteins supplied to the registry are codon optimized**

for *E. coli*, except amilCP and amilGFP that still have the native codon use of the coral *Acropora millepora.* More information about each chromoprotein can be found on the Registry of Standard Biological Parts using the table compiled by Uppsala iGEM 2013: http://2013. igem.org/Team:Uppsala/chromoproteins#l1. See also A.D. Edlund *et al.* (submitted). Optimizing 13 chromoproteins and tyrosine ammonia-lyase for use in *Escherichia coli*.

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9

Appendices

Table 1. BioBrick™ Plasmids Used in the Lab Course. BB codes of parts are those used in the Registry of Standard Biological Parts. Most chromoproteins have multiple names, so for clarity the first name given is the original one taken from the reference listed in the same row that reported its amino acid sequence.

Plasmids			Property	Resistance
1. Promoter (including RFP reporter)				
BBa_J23101			High transcription	amp
BBa_J23110			Medium-high transcription	amp
BBa_J23106			Medium transcription	amp
2. Chromoprotein gene (including RBS, not promoter) and reference				
BBa_K1033927	asFP595 (asCP, asPink)*	Lukyanov *et al.* (2000)	Pink-purple color	chloramp
BBa_K1033929	aeCP597 (aeBlue)*	Shkrob *et al.* (2005)	Blue color	chloramp
BBa_K1033930	amilCP	Alieva *et al.* (2008)	Purple-blue color	chloramp
BBa_K1033931	amilGFP	Alieva *et al.* (2008)	Yellow color	chloramp
BBa_K1033915	amFP486 (amajLime)*	Matz *et al.* (1999)	Lime green color	chloramp

(*Continued*)

Table 1. (*Continued*)

Plasmids		Property	Resistance
3. Vector backbone (including RFP reporter)			
pSB1A3		High copy	amp
pSB3A5		Medium-low copy	amp
pSB1C3		High copy	chloramp
pSB3C5		Medium-low copy	chloramp
pSB1K3		High copy	kan
pSB3K3		Medium-low copy	kan
4. Antisense sRNA gene (including promoter)			
BBa_K864440	spot42 sRNA	Antisense	chloramp
5. Fluorescent protein genes (if flow cytometer available)			
BBa_K864100	Super Yellow Fluorescent Protein 2 (SYFP2)	Yellow fluorescence	chloramp
BBa_K592100	Blue Fluorescent Protein (mTagBFP)	Blue fluorescence	chloramp

Notes: *DNA sequence was codon optimized for *E. coli* commercially; the DNA sequence can only be found by accessing the registry (using the BB code), or in A.D. Edlund *et al.* (submitted).

Tables 2–6. Lists of essential equipment and supplies for 27 students over 5 weeks (Protocols 1–11). Key manufacturers are suggested for some items, but there are usually alternatives.

Table 2.

Major Equipment	Amount
Agarose gel electrophoresis equipment	6
Bunsen burners and gas packs	8
Centrifuge for 15 mL and 50 mL tubes	1
Heating blocks	4
Ice machine	1
Magnetic stirrer	6
Microcentrifuges for 1.5 ml tubes	4
Micropipettes, 2, 20, 200, 1000 µL	10 each
Microwave oven	1
Oven/incubator (for 42°C)	1
PCR machines	7
pH meter	1
Pipetboys, battery operated, 1–20 mL	8
Scales for centrifuge tubes	1
Shakers and incubator shakers for flasks	3
Spectrophotometer, for 1 ml cuvettes	4
Spectrophotometer, Nanodrop™, for µL samples	1
Vortexer	8
Weighing balances	2

Notes:

Table 3.

Minor Equipment	Amount
Beakers, plastic	16
Goggles, safety	10
Inoculation loops	8
Inoculation spreaders, glass or metal	8
Lab coats, white	27
Magnetic stir bars	12
Magnetic stir bar retriever	1
Microcentrifuge rotor adaptors for PCR tubes	20
Notebooks, 80 pages	30
Racks, 0.2, 15, 50 mL tubes	10 each
Racks, 1.5 mL tubes	16
Scissors	4
Spray bottles, plastic, for 75% ethanol	6
Thermometers, alcohol	3
Timers	8

Notes:

Table 4.

Disposables	Amount
Bags, plastic	1 box
Cuvettes, plastic, 1 mL	3 boxes
Freezer boxes	16
Gel Extraction Kit, GeneJET (250 preps)	1
Gloves, nitrile, S, M, L	5 each
Gloves, vinyl, S, M, L	15 each
Hazardous waste boxes	5
Marker pens, black	16
Matches	8
Paper sheets, assorted colors, A4 size	1
Parafilm	2
Petri dishes, plastic (10/bag)	50 bags
pH papers, narrow-range	2
Pipettes, glass Pasteur	1 box
Pipettes, glass, 5 mL	8 boxes
Plasmid Miniprep Kit, GeneJET (250 preps)	1
Tape, clear	5
Tape, colored	5
Test tubes, 1.5 mL (500/bag)	4 bags
Test tubes, 15 mL (50/Falcon™ bag)	10 bags
Test tubes, 50 mL (25/Falcon™ bag)	10 bags
Test tubes, PCR (500/bag)	2 bags
Test tubes, PCR, in strips (120/bag)	1 bag
Tip boxes 10, 200, 1000 µL	20 each

Notes:

Table 5.

Chemicals	Amount
Agar	500 g
Agarose	200 g
Ampicillin	5 g
ATP, 100 mM, 250 µL	1
Bacto™ tryptone	500 g
Bacto™ yeast extract	500 g
Boric acid	500 g
$CaCl_2$	100 g
Chloramphenicol	5 g
dNTP, 100 mM, 4×250 µL	1
Ethanol, 95%	2 L
Glycerol	500 mL
HCl, conc.	100 mL
Kanamycin	5 g
KCl	100 g
Na_2EDTA or Na_4EDTA	500 g
NaCl	500 g
NaOH	500 g
Sybr® Safe, Invitrogen, 200 µL	2
Tris base	1 kg
Triton®X-100	100 mL

Notes:

Table 6.

Molecular Biologicals	Amount	Supplier
DpnI FD, 50 U	2	ThermoScientific™
DreamTaq™ DNA polymerase, 200 U	2	ThermoScientific™
EcoRI-HF, heat-inactivatable, 10000 U *Escherichia coli* DH5α cells	1	N.E. Biolabs™
Generuler™ 1 kb DNA ladder (5 × 50 µl)	2	ThermoScientific™
Lysozyme, egg white	1 g	Sigma-Aldrich®
Oligo VF2 5'-TGCCACCTGACGTCTAAGAA	25 nmol	Sigma-Aldrich®
Oligo VR 5'-ATTACCGCCTTTGAGTGAGC	25 nmol	Sigma-Aldrich®
Phusion® HF DNA polymerase, 100 U	1	ThermoScientific™
PstI-HF, heat-inactivatable, 10000 U	1	N.E. Biolabs™
SpeI-HF, heat-inactivatable, 500 U	2	N.E. Biolabs™
T4 DNA ligase, 1000 U	1	ThermoScientific™
T4 polynucleotide kinase, 500 U	3	ThermoScientific™
XbaI, heat-inactivatable, 3000 U	1	N.E. Biolabs™

Notes:

Table 7. Schedule for Five-Week Lab Course. Most days began with a 50-minute lecture or tutorial that was followed by lab work for the rest of the day.

Week 1	Monday	Tuesday	Wednesday	Thursday	Friday
9.15	Lecture	Lecture	Lecture	Tutorial	Lecture
10.15	Lab introduction and safety round	Make agar plates	Tutorial on BioBricks Plasmid preps	Make competent cells	Repeat experiments (if necessary)
12 Lunch					
13.15	Make solutions		BioBrick digestion Analytical agarose gel Ligations (if time allows)	Transformations	
~17:00		Practice re-streaking colonies Start overnight cultures		Plating	Re-streak colonies **Saturday:** Teacher moves plates to 4°C

Week 2	Monday	Tuesday	Wednesday	Thursday	Friday
9.15	Tutorial	Lecture	Lecture	Tutorial	Quiz
10.15	Prepare agarose gel Project designs	Plasmid preps Analytical digestion and agarose gel	Tutorial on primer design. Design primers for PCR mutagenesis	Colorimetric assays with lysozyme lysis Read sequences (arrive today or tomorrow)	Colorimetric assays with lysozyme lysis Read sequences
12 Lunch					
13.15		1st deadline to send for sequencing.	Order primers	Repeat experiments (if necessary)	
~17:00	Start overnight cultures	Bring computers on next day	Start overnight cultures		

Week 3	Monday	Tuesday	Wednesday	Thursday	Friday
9.15	Lecture	Lecture	Tutorial	Tutorial	Lecture
10.15	Receive mutagenesis primers today	Analytical gel	Ligations	Repeat experiments (if necessary)	Colony PCR
12 Lunch					
13.15	Phosphorylate primers Start mutagenic PCR	DpnI treatment O/N 2nd deadline to send for sequencing	Transformations Plating	Order primers Re-streaking	Analytical gel
~17:00					

(*Continued*)

Table 7. (*Continued*)

Week 4	Monday	Tuesday	Wednesday	Thursday	Friday
9.15	Lecture	Lecture	Tutorial	Tutorial	Lecture
10.15	Mutagenesis	Colorimetric assays	Mutagenesis	Mutagenesis	Mutagenesis
12. Lunch					
13.15		3rd deadline to send for sequencing			
~17.00	Start overnight cultures				

Week 5	Monday	Tuesday	Wednesday	Thursday	Friday
9.15	Tutorial	Lecture	Tutorial	Tutorial	Lecture
10.15	Mutagenesis	Mutagenesis	Write up lab books Prepare lab presentations	Prepare lab presentations	Lab presentations
12. Lunch					
13.15		Final deadline to send for sequencing			
~17.00					

Week 6	Monday	Tuesday	Wednesday		
9.15	Tutorial	Revision tutorial	Revision		
10.15	Complete lab books				
12. Lunch					
13.15			**FINAL EXAM**		
~17.00			End of course		

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BioBuilder: Available at http://www.biobuilder.org

iGEM Competitions; Synthetic Biology Based on Standard Parts: Available at http://www.partsregistry.org/Main_Page, http://igem.org/Start_A_Team

OpenWetWare: Available at http://www.openwetware.org

Registry of Standard Biological Parts: Available at http://parts.igem.org/Main_Page

Synthetic Biology: Available at http://openwetware.org/wiki/Synthetic_Biology

Synthetic Biology Engineering Research Center: Available at http://www.synberc.org

Synthetic Biology Open Language Visual Standard: Available at http://www.sbolstandard.org/visual

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